Eesti Maaülikool
Estonian University of Life Sciences

INFLUENCE OF CULTIVATION TECHNOLOGIES ON PATHOGENIC *FUSARIUM* SPP. OCCURRENCE AND PRODUCTION OF MYCOTOXINS IN CEREALS

ERINEVATE KASVATUSTEHNOLOOGIATE MÕJU *FUSARIUM* SPP. ESINEMISELE JA MÜKOTOKSIINIDE TEKKIMISELE TERAVILJADEL

ELINA KARRON

A Thesis
for applying for the degree of Doctor of Philosophy
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Väitekiri
filosoofiadoktori kraadi taotlemiseks
põllumajanduse erialal

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I. **Akk, E.**, Søndergaard, T. E., Sørensen, J. L., Giese, H., Kütt, M. L., Edesi, L., Lõiveke, H., Lauringson, E. 2017. The effects of nitrogen rates and intercropping on the occurrence of *Fusarium* spp. on barley kernels. *Agronomy Research* 15(S2), 1267–1275.

II. **Akk, E.**, Lõiveke, H., Edesi, L., Kütt, M.-L., Lauringson, E., Kastanje, V. 2013. Formation of the abundance of microfungi on the barley grain grown as pure and mixed crops in Central and North Estonia. *Estonian Journal of Ecology*, 62 (4), 265−275. doi:10.3176/eco.2013.4.03

III. **Karron, E.**, Runno-Paurson, E., Lõiveke, H., Islamov, B., Edesi, L., Kütt, M.-L., Talve, T., Lauringson, E., Hõrak, H., Niinemets, Ü. 2020. Application of widely used fungicides does not necessarily affect grain yield, and incidence of *Fusarium* spp. and mycotoxins DON, HT-2 and T-2 in spring barley in northern climates. *Kvasny Prumysl*, 66, 215–223. doi:10.18832/kp2020.66.215

IV. Sørensen, J. L., **Akk, E.**, Thrane, U., Giese, H., Søndergaard, T. E. 2013. Production of fusarielins by *Fusarium*. *International Journal of Food Microbiology*, 160, 206–211. doi:10.1016/j.ijfoodmicro.2012.10.016

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Samples analysing	**EA**	**EA**	**EK**	**EA**, JLS
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BI – Bulat Islamov; **EA – Elina Akk (EK-Elina Karron)**; EL – Enn Lauringson; ERP – Eve Runno-Paurson; HH – Hanna Hõrak; HL – Heino Lõiveke; HG – Henriette Giese; JLS – Jens Laurids Sørensen; LE – Liina Edesi;; M-LK – Mari-Liis Kütt; TES – Teis Esben Søndergaard; TT – Tiina Talve; UT - Ulf Thrane, VK – Veiko Kastanje; ÜN – Ülo Niinemets

ABBREVIATIONS

AreA	gene of fungi for nitrogen regulator
CFU	colony forming units
CreA	gene of fungi for carbon regulator
DNA	deoxyribonucleic acid
DON	deoxynivalenol, vomitoxin, is a secondary metabolic compound of fungi, as mycotoxin also called trichothecene
EU	European Union
EC	European Commission
FAO	Food and Agricultural Organisation
Fusarielins A-E, F, G, H	secondary metabolic compounds of fungi, mycotoxins
HT-2	toxin is a secondary metabolic compound of fungi, as mycotoxin, trichothecene A
ISSR	inter simple sequence repeat
ISRIC	International Soil Reference and Information Centre
LC-MS	liquid chromatography mass spectrometry
MAS	database for expression gene cluster
MCPA	2-methyl-4-chlorophenoxyacetic acid
MCPB	4-(4-chloro-*o*-tolyloxy) butyric acid
N	nitrogen
$NaNO_3$	sodium nitrate
PCR	polymerase chain reactor
Trichothecene A	group pf mycotoxins, including HT2 and T2
Trichothecene B	group of mycotoxins, including DON
UHPLC	ultra-high pressure liquid chromatography
UNITE	unified system for the DNA based fungal species linked to the classification
T-2	toxin is a secondary metabolic compound of fungi, as mycotoxin also called trichothecene A

1. INTRODUCTION

Cereals are worldwide major components of domestic bread basket, and feed for livestock. The export of cereals from Estonia increased from 35 million euro in 2010 to 107 million euro in 2016 (Eesti Majandus, 2017). Microbiological and toxicological purity of grain is an indicator of the quality of cereal production. Due to presence of microfungal toxins, visibly contaminated cereals cannot be used for human consumption (Jayas & Singh 2012; Varzakas, 2016). Feed cereals should also be non-toxic for livestock, but a maximum of 0.1% of *Fusarium* fungi on cereal sample is permitted (Balticagro, 2018; Scandagra, 2018; Tartu Mill, 2018). Overall, the *Fusarium* fungi in soil and on cereals are relatively well investigated (Nirenberg, 1989; Doohan *et al.,* 2003; Koch *et al.,* 2006; Medina *et al.,* 2006; Richard, 2007; Fernandez *et al.,* 2008; Chakraborty & Newton 2011; Parikka *et al.,* 2012; West *et al.,* 2012; Yli-Mattila *et al.,* 2011, 2013; Hietaniemi *et al.,* 2016). It is well known that agronomic practices such as crop rotation, tillage technology, fertilization, use of pesticides, crop species and varieties of cereals, cultivation system, and harvest conditions influence the occurrences of *Fusarium* spp. in soil and on grain (Koch *et al.,* 2006; Fernandez *et al.,* 2008; Schaafsma & Hooker 2007; Shwab & Keller 2008; Bernhoft *et al.,* 2012; Parikka *et al.,* 2012; West *et al.,* 2012). Meteorological conditions also affect the abundance of microorganisms in the soil ecosystems as well as the spread of microfungi in field conditions (Glick, 1995; Doohan *et al.,* 2003; Parikka *et al.,* 2012). In general, in more diverse soils harbouring multiple symbiotic and plant growth promoting microorganisms, the activity of pathogenic organisms is lower. For example, Perez *et al.* (2008) and Yogev *et al.* (2011) found a negative correlation between soil microbial activity and the survival of *Fusarium* spp. in crop residue and soil.

In Estonia, *Fusarium* spp. have been identified in monitored samples throughout the country since 1973 (Lõiveke *et al.,* 2003). The monitoring results showed that the majority of winter and spring cereal crop samples were contaminated by *Fusarium* fungi, including samples of rye (*Secale cereale*, 86% contamination), winter wheat (*Triticum aestivum*, 62%) and spring barley (*Hordeum vulgare*, 79%) and oat (*Avena sativa*, 87%) (Lõiveke *et al.,* 2003). Mycotoxins were identified in 41% of feed cereal samples in 2006 and in 66% of samples in 2007 (Lõiveke *et al.,*

2008). These results showed that common plant diseases, such as snow mould (*Microdochium nivale*, *Fusarium* spp.), root rot (*Cochiobolus sativus*, *Fusarium* spp., *Gaeumannomyces graminis*), leaf blights (*Cochliobolus sativus, Pyrenophora tritici-repentis, Pyrenophora avenae, Pyrenophora teres*), and head blights (*Fusarium* spp.) contributed to the spread of *Fusarium* species (Lõiveke, 2008; Jalli *et al*., 2011; Taheri, 2018).

The main *Fusarium* species such as *F. avenaceum*, *F. poae*, *F. sporotrichiella*, *F. culmorum*, *F. oxysporum*, and *F. verticilloides* dominate Estonian cereal grain (Lõiveke, 2008), while *F. graminearum*, *F. poae*, *F. sporotrichioides*, *F. langsethiae*, *F. tricinctum* are more common in other northern European countries (Hietaniemi *et al*., 2016; Yli-Mattila *et al*., 2011, 2013). In addition, the Estonian climate is more humid than in main southern and central European cereal-growing areas; especially the harvesting season is often wet such that the harvested grain needs extensive drying to remove excess water before storage. Such humid conditions can create ideal conditions for *Fusarium* spread and development on grain, implying an increased risk of mycotoxin contamination. Indeed, there is evidence that the mould count and the occurrence of *Fusarium* spp. increases with amount of precipitation and precipitation frequency during the flowering and pre-harvest time of cereals (Lõiveke *et al*., 2008). Although weather conditions have a major influence on *Fusarium* head blight spreading, but it is not well understood if different *Fusarium* species produce mycotoxins under comparable environmental conditions. Climatic changes such as increasing winter soil temperatures modify the composition of toxigenic fungi and their interactions with agricultural crops, thereby creating conditions for higher contamination of mycotoxins in final products (Moretti *et al.,* 2019; Lukas *et al.,* 2018). Thus, mycotoxins levels in food and feed cereal must be consistently monitored. More diversified cultivation technologies and agricultural practices may decrease the contamination of grain with mycotoxigenic fungi and mycotoxins.

The main aim of the thesis was to gain an insight into the spread of *Fusarium* spp. under Nordic climatic conditions as driven by agricultural practices (N addition rate, crop cultivation practices, intercropping) and, characterize the spread of key mycotoxins and ways of controlling *Fusarium* spread and mycotoxin formation.

2. REVIEW OF LITERATURE

2.1. *Fusarium* fungi and mycotoxins

Fusarium is a large genus of filamentous fungi which was described in 1809 (Link, 1809). It is one of the most important groups of plant pathogenic fungi because it includes human pathogenic fungi. The number of recognized species within the genus is considerable; there are more than 1,700 names with some association to *Fusarium* in MycoBank database (MycoBank, 2020). The *Fusarium* fungi include common moulds found in fields around the world living saprotrophically on crop residues by degrading organic matter (Sutton, 1982; Kosiak *et al.,* 2004; Popovski & Celar 2013). They overwinter on plant debris in the soil (Sutton, 1982; Pereyra & Dill Mackey, 2008; Fernandez *et al.,* 2008). The main host plants of *Fusarium* spp. are cereals and solanaceous crops such as potato (*Solanum tuberosum*) and tomato (*S. lycopersicum*) (Nirenberg, 1989), but *Fusarium* spp. can infect most crops. Summerell (2019) noted that according to the American Phytopathology Society website, 83 of 108 crop plant species in the database have one or more *Fusarium*-caused diseases which impact their production. In field conditions, *Fusarium* spp. also contribute to infection of crops by other microfungi. The occurrence of *Fusarium* fungi in small grain crops causes poor grain quality because of mycotoxin contamination and yield reduction (Medina *et al.,* 2006; Richard, 2007; Chakraborty & Newton 2011). *Fusarium* spp. infection leads to shrunken kernels (Richard, 2007). Mycotoxins are toxic compounds naturally produced by moulds (fungi), including *Fusarium* moulds, as secondary metabolites, (WHO, 2018). They occur in food and feed crops around the world causing serious health problems for humans (Bryden, 2007) and livestock (Pinotti *et al*., 2016).

Infection with *Fusarium* spp. spores in the field takes place when the cereals are in the anthesis stage and the pollen is contaminated with *Fusarium* spp. spores (Tekle *et al.,* 2012; Persson *et al.,* 2017). However, infection is also possible at milk to early dough stages in spring wheat (Del Ponte *et al.,* 2007). The spread of *Fusarium* spp. is affected by numerous factors, such as agro-meteorological conditions and agronomic practises (Parikka *et al.,* 2012). The infection usually occurs when air temperature is 10–35 ºC and when the plant tissue has been wet at least 24 consecutive hours (Rossi *et al.,* 2001). *Fusarium* spp.

produce mycotoxins in high moisture (20%) conditions (Logrieco *et al.*, 2003). In favourable conditions, *Fusarium* spp. may produce one or more mycotoxins, with different levels of toxicity (Bottalico & Perrone 2002). *Fusarium* spp. abundance and suppression of infections are related to soil microbial diversity and activity (van Bruggen & Semenov 1999; Borrero *et al.,* 2004; van Bruggen *et al.,* 2006; Liu *et al.,* 2007; Perez *et al.,* 2008; Bonanomi *et al.,* 2010; Yogev *et al.,* 2011).

Approximately 20 *Fusarium* species cause Fusarium head blight (FHB) and contamination of cereal grain by mycotoxins, including frequently occurring species *F. avenaceum*, *F. culmorum*, *F. poae*, *F. sporotrichioides,* and *F. graminearum* (Keith & Lévesque 2004; Wagacha & Muthoni 2007; Fernandez *et al.,* 2008; Yli-Mattila, 2010; Parikka *et al.,* 2012; Fredlund *et al.,* 2013). *F. graminearum, F. culmorum, F. poae, F. sporotrichioides, F. tricinctum, F. avenaceum and F. langsethiae* are most common species producing mycotoxins DON, T-2, HT-2, and nivalenol on the grain in northern Europe (Yli-Mattila *et al.,* 2011, 2013; Hietaniemi *et al.,* 2016). In previous studies in Estonia, Lõiveke (2008) found that leaf blights (*Cochliobolus sativus, Pyrenophora tritici-repentis, Pyrenophora avenae, Pyrenophora teres*), root rot (*Cochiobolus sativus*, *Fusarium* spp.*, Gaeumannomyces graminis*), and snow mould (*Microdochium nivale*, *Fusarium* spp.) in cereals crops were primarily caused by *Microdochium nivale*, *F. avenaceum*, *F. culmorum*, *F. solani*, *F. oxysporum* and *F. verticilloides*.

Over 170,000 natural fungal compounds have been discovered (Gruber-Dorninger *et al.*, 2017), and more than 300 mycotoxins have been identified and reported in crops (Alshannaq & Yu 2017). The most common mycotoxins are aflatoxins (produced by *Aspergillus flavus* and other *Aspergillus spp.*), fumonisins (produced by *Fusarium* spp.), trichothecenes (produced by different species of *Fusarium*), zearalenone (produced by different species of *Fusarium*), citrinin (produced by *Penicillium* spp. and *Aspergillus* spp.), patulin (produced by different species of *Penicillium*), ergot alkaloids (produced by *Claviseps purpurea*), ochratoxin A (produced by *Aspergillus ochraceus*) (Rocha *et al.,* 2014), enniatins (*Fusarium* spp.), beauvericin (*Fusarium* spp.), moniliformin (*Fusarium* spp.), fusaproliferin (*Fusarium* spp.), fusaric acid (*Fusarium* spp.), culmorin (*Fusarium* spp.), butenolide (*Fusarium* spp.), sterigmatocystin (*Aspergillus* spp., *Bipolaris* spp., *Emericella* spp., *Penicillium* spp., *Chaetomium* spp., *Botryotrichium* spp., *Humicola* spp.), mycophenolic acid (*Penicillium brevicompactum*), alternariol

and alternariol monomethyl ether (*Alternaria spp.*) and tenuazonic acid (*Alternaria* spp.) (Gruber-Dorninger *et al.,* 2017).

Trichothecenes are compounds with a tetracyclic sesquiterpenoid 12, 13-epoxytrichothec-9-en structure. An epoxide group between C_{12} and C_{13} gives the toxic effect (EFSA Journal, 2017). Trichothecenes are classified in four groups, A, B, C, and D, whereas trichothecenes from group A and B most commonly contaminate food and feed (EFSA Journal, 2017). The difference between A and B type trichothecenes is at the C8 position, where the B type has a carbonyl group and the A type has an ester side chain or no side chain (Ueno, 1984; Döll & Dänicke 2011).

The review article by Sobrova *et al.,* (2010) describes deoxynivalenol (DON), also known as vomitoxin, a type B trichothecene, with chemical name 12,13-epoxy-3α,7α, 15-trihydroxytrichothec-9-en-8-on. Its toxicity is due to three free hydroxy groups. Mycotoxin DON is heat-stable even at temperatures 170–350 ºC, dissolves in water and organic solvents (ethanol, chloroform), but not in oils. Mycotoxin DON can contaminate all unprocessed cereal species and products produced from cereals, such as bread, noodles, flour, beer, and malt (Sobrova *et al.,* 2010). The human poisoning symptoms are vomiting, nausea, diarrhoea, fever, and headache (Sobrova *et al.*, 2010). Animal symptoms include vomiting, diarrhoea, nausea and loss of appetite, and loss of body weight (Rocha *et al.,* 2014).

Toxins T-2 and HT-2 are type A trichothecenes; HT-2 toxin is an acetylated form of T-2 toxin (Nathanail *et al.,* 2015). The chemical name of T-2 is 3α-hydroxy-4β,15-diacetoxy-8α-(3-methylbutoxy) -12,13-epoxy-trichothec-9-ene and that of HT-2 is 3α,4β-dihydroxy-15-acetoxy-8α-(3-methylbutoxy)-12,13-epoxy-trichotec-9-ene (Schmidt *et al.,* 2018). Contamination of feed with toxins T-2 and HT-2 affects blood cells, immune system, digestive system, liver, nervous system, skin, pregnancy, egg and milk production, and also may cause animal death (Kalantari & Moosavi 2010; Pereira *et al.,* 2019).

In order to avoid serious health problems in humans and animals, maximum tolerated toxin levels have been established. Table 1 gives mycotoxin levels for DON, T-2, and HT-2 in food and feed based on European Commission recommendations 1881/2006 (EUR-Lex, 2006) and 2013/165 (EUR-Lex, 2013). The permitted levels of toxins vary for

different toxins and different levels have been established for different cereals and level of processing.

Studies conducted in Europe, Scandinavia, and North-America showed that DON was present in 58–91% of barley (*Hordeum vulgare*) grain samples, while T-2 was present in 50–61% and its deacetylated form, HT-2, was present in 12–50% of barley grain samples (Pettersson, 1996; Perkowski *et al.*, 2003). DON infestation varied between fields and years in oat (*Avena sativa*) (Persson *et al.*, 2017). In Estonia, mycotoxins were present in 30% of food cereal samples and in 58% of feed cereal samples in 1999 (Lõiveke *et al.*, 2004) in 41% of feed cereal samples in 2006 and in 66% of samples in 2007 (Lõiveke *et al.,* 2008).

Table 1. Maximum permitted levels of mycotoxins DON, T-2, and HT-2 in cereals and cereal products for human food and animal feed based on European Commission recommendations 1881/2006 (EUR-Lex, 2006) and 2013/165 (EUR-Lex, 2013).

DON in food	**µg kg^{-1}**
Unprocessed cereals (excluding durum wheat, oats, maize)	1250
Unprocessed durum wheat and oats	1750
Unprocessed maize	1750
Cereals for direct human consumption (flour, bran, germs, pasta, snacks, bread, flakes)	750
Baby and infant food	200

T-2+HT-2 in food	**µg kg^{-1}**
Barley, malting barley, maize	200
Wheat, rye and other cereals	100
Oats with husk	1000
Oats for direct human consumption	200
Other cereals for direct consumption	50
Maize for direct consumption	100
DON in feed	**µg kg^{-1}**
Cereals and cereal-based feed	8000
Maize by-products	120000
Complementary, complete feedstuff	5000
Complementary, complete feed for pigs	900
Complementary, complete feed for calves, lambs, kids	2000
T-2 + HT-2 in feed	**µg kg^{-1}**
Oat products with husks	2000
Other cereals products	500
Compound feeds	250

2.2. Factors influencing the level of *Fusarium* spp. and mycotoxins contamination in cereals

Cultivation practices, tillage technology, crop rotation and pre-crop use and type, use of pesticides and time of harvesting are the agronomic factors and the climate and soil type the ecological factors affecting *Fusarium* spp. spread (Bullied *et al*., 2006; Bernhoft *et al*., 2012). A combination of agronomic factors affect the mould composition on cereal grains (Koch et al., 2006; Fernandez et al., 2008; West et al., 2012), but the formation of mycotoxins is particularly affected by ecological factors (Schaafsma & Hooker 2007; Shwab & Keller 2008; Bernhoft *et al.,* 2012). The composition and development of *Fusarium* fungi on plant residue in the soil depends on a combination of many conditions, of which environmental conditions, particularly the precipitations at the time of ripening, play a main role (Magan *et al.,* 2002; Pirgozliev *et al.,* 2003; *et al.,* 2010; Gaurilčikienė *et al.,* 2011).

2.2.1. Agronomic factors

Plant cultivation technology, including soil tillage practises may change the microbiology in soil and thereby plant health (Smith *et al.,* 2016) as different soil tillage practices influence the soil biological processes carried out by microorganisms by changing plant residue distribution and soil physical properties (Janušauskaite *et al.,* 2013; Leplat *et al*., 2013). However, the available evidence on the impact of different agricultural practices on *Fusarium* spp. occurrence is controversial. In one study, different tillage technologies (ploughing, minimum tillage and zero till) did not affect *Fusarium* spp. frequency and mycotoxin content on cereal (Bernhoft *et al.* 2012). Other researchers have found that minimum tillage does increase the risk of infection, but nevertheless, whether conditions exert the main control on infection (Dill-Macky & Jones 2000; Bateman *et al.,* 2007; Leplat *et al*., 2013). The contamination with moulds and the concentration of mycotoxins can be lower in organic than in conventional farming system (Bernhoft *et al.,* 2010; Bernhoft *et al.,* 2012). Karlsson *et al*., (2017) found that higher fungal community in winter wheat phyllosphere was in organic farming field compare in conventional farming field. The reason might be explain by longer varied crop rotation, by the no use synthetic pesticide (Sapkota *et al*., 2015) and fertilizers, by no use herbicide glyphosate (Fernandez *et al*., 2009), also soil management by plowing (Edwards, 2009b; Bernhoft *et al*., 2010).

A cereal-dominated crop rotation enhanced contamination of harvest with *Fusarium* fungi and mycotoxins DON, HT-2 (Edwards, 2004; Bernhoft *et al.,* 2012). Using maize (*Zea mays*) as the preceding crop of cereals is a risk factor because maize debris is favourable for harbouring *Fusarium*, specifically for *F. graminearum*, and spreading it to the subsequent crop (Dill-Macky & Jones 2000; Pereyra *et al.,* 2004; Beyer *et al.,* 2006; Bateman *et al.,* 2007; Pereyra & Dill-Macky 2008; Blandino *et al.*, 2010; Müller *et al.*, 2010).

Fertilization may affect the contamination of *Fusarium* and presence of mycotoxins on grain as high levels of N, over 80 N ha^{-1}, increase the density of plant canopy, and thereby also influence the disease development in crops (Lemmens *et al.,* 2004; Dordas, 2008; Suproniene *et al.,* 2011b). Fungal mycotoxin production is influenced by soil carbon and nitrogen (N) sources (Jiao *et al.,* 2008; Min *et al.,* 2012; Sørensen & Giese 2013). Infestation of *F. graminearum* increased when animal and green manure was used (Bernhoft *et al.,* 2012). However, Perez *et al.,* (2008) found that green manure application on buckwheat (*Fagopyrum esculentum*) field increased the abundance of *F. graminearum* antagonist streptomycetes and bacteria in soil.

The use of chemical control methods, such as fungicide spray, at the cereal growth stage is well studied and is recommended for prevention of mycotoxin accumulation on grain (Wegulo *et al.,* 2015). In winter wheat (*Triticum aestivum*) field trials the spraying of fungicides was effective against individual *Fusarium* species; tebuconazole reduced the content of mycotoxins DON, T-2, and zearalenone (Suproniene *et al.,* 2011a). The active ingredients of the triazole group of fungicides were most effective against *Fusarium* fungi (Sooväli *et al.,* 2017). However, in some research the opposite effect has been found and the use of pesticides has increased the risk of *Fusarium* infestation and mycotoxin contamination. Bernhoft *et al.*, (2012) found that spraying with fungicides (azoxystrobin combined with fenpropimorph and propiconazol combined with trifloxystrobin) increased *Fusarium* abundance and mycotoxin content, while spraying with herbicides (tribenuron-methyl and MCPA (2-methyl-4-chlorophenoxyacetic acid)) reduced contamination. At the same time, glyphosate use increases *Fusarium* abundance in the soil and cereal contamination (Fernandez *et al.,* 2009). The controversial results might reflect impacts of plant phenological stage at the time of spraying, site-

specific differences and weather differences, but the available information is limited to gain conclusive insight into study-to-study differences.

The use of *Fusarium*-resistant cultivars is the most economical, effective, and environmentally friendly method of control (Zhang *et. al.,* 2011). With selection of disease-resistant varieties, it is possible to significantly reduce *Fusarium* disease incidence and DON accumulation on grain (Schaafsma, 2001; Champeil *et al*., 2004; Fernandez *et al.,* 2005; Gilbert & Tekauz 2011). Three types of genetic resistance have been found: resistance to initial infection, resistance to spread of the pathogen, and resistance to accumulation of mycotoxin (Schroeder & Christenson 1963; Miller *et al.,* 1985; Hollins *et al.,* 2003). Thus, cereal breeders have been searching for resistant varieties and they have been successful in breeding several resistant winter and spring wheat (*Triticum aestivum*) lines (Mesterházy, 2003; Parikka *et al.,* 2012). Oat (*Avena sativa*) breeding has focused on crosses with wild oats to enhance the oat resistance (Bjørnstad & Skinnes 2008; Parikka *et al.,* 2012). Nevertheless, broad-spectrum sustained resistance to different *Fusarium* species has been difficult to achieve, implying that once resistant cultivars can be infected by new *Fusarium* strains.

2.2.2. Environmental factors

Environmental factors such as temperature, humidity, precipitation, and soil type strongly affect the spread of *Fusarium* populations during plant development (Xu, 2003, Parikka *et al.,* 2012; Popovski & Celar 2013). It is expected that under changing weather conditions, the spread of *Fusarium* spp. and share of mycotoxin-contaminated cereals in world agriculture will increase (Moretti *et al.,* 2019).

The effect of temperature on *Fusarium* species has been demonstrated by *in vitro* laboratory tests (Brennan *et al.,* 2003; Dufault *et al*., 2006). The temperature at which *F. avenaceum*, *F. culmorum,* and *F. graminearum* inhibited coleoptile growth was 20–25 ºC (>89.3% reduction), while *F. poae* and *M. nivale* inhibited coleoptile growth at 10–15 ºC (>45.6% retardation), compared to non-inoculated control seedlings (Brennan *et al.,* 2003). Grain contamination with *F. graminearum* and *F. langsethiae* positively correlated with high air temperatures (16–25 ºC) in cereals during the pre-harvest period (Dufault *et al*., 2006; Bernhoft *et al.,* 2012),

but DON content decreased as air temperature increased before harvest (Doohan *et al.,* 2003).

The amount of precipitation in the post-flowering period affected the occurrence of *Fusarium* and mycotoxin content in oat (*Avena sativa*) and wheat (*Triticum aestivum*) (Hjelkrem *et al.,* 2017). There is evidence that a large amount of precipitation at that developmental stage is favourable for infestation by *Fusarium* fungi (Bernhoft *et al.,* 2012). At barley (*Hordeum vulgare*) ripening stage, a high rainfall created favourable conditions for contamination by *Fusarium* spp. (Roháčik & Hudec 2007). In Estonia, anthesis, grain filling, and harvesting for spring cereal occur from July to September, and these growth stages are often coupled with the strongest rainfall events, although there is a large year-to-year variability (Estonia's Seventh National Communication…, 2017).

Soil type also influences the occurrence of *Fusarium* species on cereals grains. Incidence of *F. graminearum* was lower on cereals grown in clay-rich soil than on a sandy (Bernhoft *et al.,* 2012). Soil humus content plays an important role in plant growth and the composition of microorganisms (Bonanomi *et al*., 2007). In laboratory tests, humic substances, such as humic acids and fulvic acids, mainly influenced *F. culmorum* mycelium growth, but affected less spore germination (Moliszewska & Pisarek 1996). The results of trials in eight field locations in Belgium showed that the *Fusarium* species complex was different in soil samples, on crop residues, on gramineous weeds, and on wheat ears (Landschoot *et al.,* 2011). Thus, both soil and plant factors determine the degree of *Fusarium* infection, and it is difficult to directly link soil characteristics to plant infection severity.

3. AIMS AND HYPOTHESIS OF THE STUDY

Fusarium spp. are the most common and pervasive plant pathogens and cause widespread crop losses. Several *Fusarium* species can produce mycotoxins, which cause serious diseases in animals and humans. Grain contamination with *Fusarium* spp. and mycotoxins is affected by the combination of several agronomic and environmental factors. So far, the main approach to prevent and reduce cereal grain contamination is by the selection of suitable agronomic practises and timing of control treatments. Although the impact of agronomic practices on *Fusarium* spp. composition and abundance and mycotoxin production on grain and on *Fusarium* spp. composition and abundance in the soil has been studied in several countries, the results are not directly transferable to different climatic conditions. There is a lack of information on this topic in the Northern Baltic region where cereal harvest season is typically combined with wet weather that creates ideal conditions for the spread of *Fusarium*. Thus, the purpose of this thesis is to determine whether agronomic practices including intercropping, N fertilization and fungicide usage prevent or decrease the contamination of cereal grains with *Fusarium* fungi, mould, yeast and mycotoxins in the Northern Baltic region.

The specific aims of the thesis are:

1) to examine and compare the composition of *Fusarium* species in spring barley grains grown with mineral N fertilizer and in pea-barley intercropping (**I**);

2) to compare the influence of the rate of N application, location, and weather on the abundance of moulds, yeasts, and *Fusarium* spp. on barley grain (**II**);

3) to investigate the effect of fungicide treatment on the incidence of *Fusarium* spp. and mycotoxins DON, HT-2, and T-2 in spring barley (**III**);

4) to examine the impacts of source of carbohydrates, time, acidity and temperature of the environment on the production of mycotoxins in *Fusarium* spp. (**IV**).

Hypotheses:

Paper I hypothesized that intercropping barley with field pea would decrease the occurrence of *Fusarium* spp. in spring barley grain compared to spring barley grown with mineral fertilizer.

Paper II hypothesized that N fertilization of spring barley would increase the abundance of moulds, yeasts and *Fusarium* spp. on barley grain.

Paper III hypothesized that the treatment with different fungicides would decrease the occurrence of *Fusarium* spp. and mycotoxin production in spring barley grain in fungicide-specific manner and the effects differ among years with different weather.

Paper IV hypothesized that the capacity of *Fusarium* spp. to produce mycotoxins depends on the available nitrogen and carbon sources for fungal growth and that the mycotoxin production capacity is also affected by environmental conditions.

4. MATERIALS AND METHODS

4.1. Experiment location and design

4.1.1. Field experiment sites, design, and sample collection

Field experiments were conducted in 2009–2010 (**I**, **II**) in Olustvere, Central Estonia (58º33´N, 25º34´E) on sod-podzolic, *Podzoluvisol* (FAO, ISSS, ISRIC. 1998) and on rendzina soil, *Cambisol* (FAO, ISSS, ISRIC. 1998) (**II**). In 2012–2014 (**III**) they were conducted on gley soil, *Gleysol* (FAO, ISSS, ISRIC. 1998) in North Estonia (59º18´N, 25º39´E).

Three field experiments were conducted to collect grain samples for analyses. The experimental plots were ploughed each autumn and the treatment plots were randomized 25 m^2 plots (**I**, **II, III**). The field experiments with spring barley (*Hordeum vulgare* L.), variety 'Anni', and field pea (*Pisum sativum* L.), variety 'Clarissa', (**I, II**) were in Central and North Estonia, while the third field experiment with spring barley (*Hordeum vulgare* L.), variety 'Maali' (**III**), was carried out in North Estonia.

The first two experiments (**I, II**) had four treatments: 1) barley with 120 kg ha^{-1} N, 2) barley with 60 kg ha^{-1} N, 3) barley without N, and 4) barley-pea intercropping. A complex mineral fertilizer with 12.5 kg ha^{-1} N was applied at sowing in all experiments. In the fertilization treatments, ammonium nitrate was applied at the end of the leaf development stage (**I, II**).

The third experiment (**III**) had three treatments and a control: 1) barley unsprayed as control, 2) barley sprayed with active ingredient tebuconazole, 3) barley sprayed with a commercial mix of active ingredients prothioconazole, spiroxamine, and tebuconazole, 4) barley sprayed with a commercial mix of active ingredients fenpropidin and propiconazole. Fungicides were applied at flowering (**III**). A complex mineral fertilizer with 12.5 kg ha^{-1} N was applied at sowing in all experiments. Ammonium nitrate was applied at a rate of 60 kg ha^{-1} N at the beginning of stem elongation (**III**).

Herbicides Butoxone (active ingredient MCPB) (**I**, **II**) and MCPA (active ingredient 2-methyl-4-chlorophenoxyacetic acid) (**III**) were used for weed control. After harvesting, the grain was dried to 14% moisture content, cleaned, and 1.5 kg samples were taken from each treatment (**I**, **II**, **III**). The abundance of moulds, yeasts and *Fusarium* spp. were determined using dilution methods in the Laboratory of Pant Health and Microbiology in the Agricultural Research Centre (**II**).

4.1.2. Collection and culture of *Fusarium* spp.

Fifteen strains of *Fusarium* species: *F. avenaceum*, *F. cerealis, F. culmorum, F. graminearum, F. equisety, F. langsethiae, F. oxysporum, F. poae, F. proliferatum, F. pseudograminearum, F. sambucinum, F. solani, F. sporotrichioides, F. tricinctum, F. venenatum*, were evaluated for their ability to produce mycotoxins fusarielins in a laboratory experiment at Aarhus University in Denmark (**IV**). The *Fusarium* strains were grown one week on Czapec Dox agar (CZ). Mycelia were transferred to malt extract agar (MEA), oatmeal agar (OA), potato dextrose agar (PDA), yeast extract agar (YES), or Czapec Dox agar (CZ). The plates with mycelia on different agars were incubated at 25 ºC in the dark for two weeks. A *Fusarium graminearum* strain was used to examine the effect of different carbon and N sources, different media acidity (pH), air temperature, and time on the production of mycotoxins fusarielins (**IV**).

4.1.3. Isolation and identification of *Fusarium* spp. on grain (I, III)

Barley grains were sterilized with 1% of sodium hypochlorite (NaOCl) and rinsed twice with sterile, distilled water. Following the procedure of Abildgren *et al.* (1987), the grains were placed on Petri dishes containing Czapec Dox agar. The plates were incubated at 20 ºC with dark/light (8 h/16 h) cycle. After seven days of incubation, *Fusarium* spp. contaminated grains were counted (**I**, **III**). *Fusarium* spp. on grains were transferred to Potato Dextrose Agar (PDA) and inoculated in the dark for 14 days at 28 ºC (**I**). After incubation, DNA from the isolated fungi was purified with the Ultra Clean Microbial DNA Isolation Kit, according to the manufacturer´s protocol. The internal transcribed spacer (ITS) region of the rDNA gene (S) was amplified using fungal specific primers for identification of fungi (White *et al.* 1990). A thermal cycler profile was used for denaturation at 98 ºC for 5 min followed by

30 cycles of amplification for denaturation at 98 ºC for 1 min, primer annealing at 52 ºC for 1 min, extension at 72 ºC for 5 min, followed by cooling at 4 ºC. Next 35 µl of PCR product was taken and cleaned further with a Qiaquik PCR Purification Kit. The sample submission guide of EUROFINS MWG Operon was followed to prepare DNA for sequencing of the PCR products. The obtained DNA sequences were analysed using the UNITE database (Kõljalg *et al.,* 2013). The BLAST searching system at GenBank sequence database (BLAST, 2020) was used to identify the fungal strains (**I**).

4.1.4. Detection of fusarielins (IV) and mycotoxins DON, HT-2, and T-2 (III)

Gas chromatography mass-spectrometry (GC-MS, Agilent 7890A and Agilent 5975C) was used to detect mycotoxins DON, HT-2, and T-2 (**III**). DON, HT-2, and T-2 were analysed according to the trichothecene analysis method (Saastamoinen and Saloniemi, 1997). The detection threshold for mycotoxins was 21.0 ± 0.5 µg kg^{-1}. Three replicate injections were taken from each variant for mycotoxin analysis (**III**).

All *Fusarium* spp. strains were grown on five diverse media to examine possible regulation patterns and increase the chance of activating silent fusarielin gene clusters. Extraction of fusarielins was done with six plugs from two-week-old cultures which were first extracted ultrasonically for 30 min with 1 mL ethyl acetate. The extracts were evaporated to dryness under vacuum and re-dissolved ultrasonically in 600 µL methanol. One mL liquid cultures were extracted with 3 mL acetonitrile/water/acetic acid by using a Vibracell VC130 sonic water. One mL of the extract was centrifuged and transferred to a 2 mL vial (**IV**).

Initial screens for the presence of fusarielins A, F, G, and H in the selected *Fusarium* species were performed by liquid chromatography mass-spectrometry (LC-MS), quantity of fusarielins by Ultimate 3000 Ultra High Perfomance Liquid Chromatography (UHPLC, Idstein) (**IV**).

Fusarium graminearum (strain IBT 9203) was grown with various carbon sources: two aldopentoses (arabinose and xylose), two betahexoses (fructose and sorbose), three aldohexoses (galactose, mannose and glucose), four disaccharides (lactose, maltose, sucrose and trehalose), six

polysaccharides (dextrin, glycogen, and xylan), and starch from corn, rice and potato (**IV**).

Fusarium graminearum strain IBT 9203 was also grown on CZ with fructose as the carbon source and $NaNO_3$ as a N source to examine the effects of varying pH levels (4, 5, 6, 7, 8 and 9, accuracy of pH establishment ±0.1 pH units in all cases), temperatures (15, 20, 25, 30 and 35 ºC), fructose levels (0, 15, 30, 60, 120 and 240 ng mL^{-1}) and time (6, 10, 14, 18, 22 and 26 days) on fusarielin production (**IV**).

4.2. Weather conditions

The meteorological data were obtained from observation points of the Estonian Meteorological and Hydrological Institute (**I**, **II**) and the weather station of the field experiment in North Estonia (**III**). For the lab experiment in publication **IV**, controlled-environment conditions are reported in the paper.

The weather conditions from May to August in 2009 and 2010 were different in Central (**I**, **II**) and North Estonia (**II**). The air temperatures were suitable for plant development in both locations in 2009. However, the average air temperature was 2.4 ºC warmer in 2010 compared to 2009. The precipitation was variable each year in North and Central Estonia. In Central Estonia it rained 20% more in 2009 and 14% more in 2010, compared to the long-term average precipitation. In North Estonia it rained 26% less in 2009 and 33% less in 2010, compared to the long-term average precipitation.

The weather conditions were variable in North Estonia during 2012–2014 (Table 1, **III**). During the 2012 growing season the weather was very wet and cool. Weather conditions were hot and dry in the 2013 growing period, but in August when barley matured, it rained much more than the long-term average (51% more). In 2014, the air temperature and the amount of precipitation varied in each month. June was cool and wet followed by a hot and dry July while August was hot with a normal amount of precipitation (**III**).

4.3. Data analysis

Analysis of variance (ANOVA, SAS 2002) was used (**I, II, III**) to test for the effects of treatments on the incidence of *Fusarium* spp. on grains. The concentrations of toxins T-2 and HT-2 were summarized since toxin T-2 can be metabolized to toxin HT-2, and both co-occurred in the grains (Hjelkrem *et al.*, 2018; Nathanail *et al.*, 2015) (**III**). In all statistical tests, the level of significance was $p<0.05$ (**I, II, III**).

The fusarielin gene cluster of *Fusarium* species were analysed with the databases MAS 5.0 and detected fusarielins with the database Insilio (**IV**).

5. RESULTS

5.1. Effects of ammonium nitrate and barley-pea intercropping, and effect of spraying with fungicides (I, II, III)

Incidence of *Fusarium* fungi on barley was variable in 2009 and 2010 in Central Estonia conditions (Table 2, **I**). Twelve species of fungi were identified on spring barley grain. In 2009 *Fusarium* fungi were isolated from 38% of grain and other pathogenic fungi from 62% of grain (**I**). Different results were seen in 2010 when *Fusarium* spp. were isolated from 96% of barley grain (**I**).

The N application rate and pea-barley intercropping influenced the incidence of microfungi on barley grain (Figure 1, **I**). The lowest incidence of microfungi (30%) was found in barley grain from the pea-barley intercropping ($p<0.05$). The incidence of microfungi on barley grain was 54% in N40, 38% in N60, 48% in N120 ($p<0.05$) (Figure 1, **I**).

The two most frequently occurring species were *Fusarium avenaceum* and *Fusarium poae* (Table 2, **I**). *Fusarium poae* and *F. sporotrichioides* were always present in barley grain which received top dressing of ammonium nitrate; even at the lowest N level (N40). However, only *F. avenaceum* was found in barley grain from the intercropping treatment ($p<0.05$) (Table 3, **I**).

The abundance of moulds, yeasts, and *Fusarium* spp. on barley grain varied between years and locations (Figures 1 and 2, **II**). The average abundance of mould was four time higher in Central Estonia than in North Estonia ($p<0.05$) (Figure 2b, **II**). The N levels and barley-pea intercropping had no effect on the incidence of moulds, yeasts, or *Fusarium* spp. in barley grain ($p>0.05$) (Figure 2c, **II**). The abundance of *Fusarium* spp. was low, only 1.6% of the abundance of moulds and yeasts (**II**).

In 2009 moulds were the most common microfungi group in barley grain, while in 2010 yeasts dominated (species not determined) (**II**). However, the total occurrences of moulds, yeasts, and *Fusarium* spp. was higher in 2010 than 2009 ($p<0.05$) (Figure 2a, **II**). The development of yeasts was enhanced by 2–3 degree higher air temperatures in 2010 (**II**). The abundance of moulds in barley from Central Estonia was four

times higher and the abundance yeasts two times higher compared with barley from North Estonia (p<0.05) (Figure 2b, **II**).

Treatment of spring barley with fungicides reduced the occurrence of *Fusarium* fungi (p<0.05) (Table 3, **III**) but mycotoxins still developed in the grain (Table 4, **III**). *Fusarium* fungi occurred on 3.8% of barley grains in 2012 and there was no significant difference in the incidence of *Fusarium* spp. between fungicide treatments (p>0.05) (Table 3, **III**). The incidence of *Fusarium* spp. was 15.3% in 2013 and 14.0% in 2014. The fungicide with active ingredients fenpropidin and propiconazole had the lowest incidence of *Fusarium* spp., 4% in 2013 and 5% in 2014 (p<0.05) (Table 3, **III**).

5.2. Mycotoxins DON, HT-2, and T-2 in spring barley grain (III)

The year and fungicide treatment interaction had a significant effect on the incidence of DON, HT-2, and T-2 (p<0.001) (Table 4, **III**). However, the concentration of all studied mycotoxins was low. Higher precipitation and low air temperatures during the growing period in 2012 (Table 1, **III**) increased contamination with DON, HT-2, and T-2 (Table 4, **III**) in all treatments. However, the concentration of DON was significantly greater (p=0.001) in the untreated control (73.3 μg kg^{-1} barley grain) and in the treatment with three active ingredients, prothioconazole, tebuconazole and spiroxamine (69.7 μg kg^{-1}), compared with the treatments of tebuconazole and fenpropidin with propiconazole (Table 4, **III**). In 2012 HT-2 and T-2 concentrations were significantly higher in the treatment with active ingredients fenpropidin with propiconazole (62.3 μg kg^{-1}) (p=0.001) compared to the treatments with prothioconazole, tebuconazole and spiroxamine (25.6 μg kg^{-1}), tebuconazole (26.2 μg kg^{-1}), and the untreated control (27.5 μg kg^{-1}) (Table 4, **III**). In 2013, a dry and warm growing season combined with rainy and warm weather during maturation (Table 1, **III**) increased contamination by DON in all treatments; however, the variation between treatments was significant (p=0.009) (Table 4, **III**). The highest concentration of DON, 63.1 μg kg^{-1} was found in the treatments with prothioconazole, tebuconazole and spiroxamine and fenpropidin with propiconazole (Table 4, **III**). HT-2 and T-2 were not found in barley grains in 2013 (Table 4, **III**). 2014 was a warm year with normal precipitation (Table 1, **III**), and DON, HT-2, and T-2 were detected in the untreated control and the tebuconazole treatment (p<0.001) (Table 4, **III**).

5.3. Impact of nitrogen and carbon sources (IV)

The ability of fifteen *Fusarium* spp. and 42 strains of *Fusarium* to produce the mycotoxin fusarielin were evaluated. Only two *Fusarium* species produced fusarielins. *Fusarium tricinctum* produced fusarielin A and *F. graminearum* produced fusarielins F, G, and H. The two *Fusarium* strains prefer different media to produce fusarielins (Table 2, **IV**). The strains of *F. tricinctum* IBT 2952 and IBT 8166 produced the greatest concentration fusarielin A, 33.7 and 27.0 μg mL^{-1}, respectively, on the CZ media (Table 2, **IV**). The *F. tricinctum* strain IBT 400088 produced the largest amount of fusarielin A on the YES media, 2.2 μg mL^{-1} (Table 2, **IV**). The three strains of *F. graminearum*, NRRL 31084, IBT 1955, and IBT 9203, produced the greatest concentrations of fusarielin H on the YES media, 10.3, 36.9, and 77.0 μg mL^{-1}, respectively (Table 2, **IV**).

The impact of eighteen carbohydrates as carbon sources and two N sources (nitrate and arginine) on mycotoxin formation were tested in *F. graminearum* strain IBT 9203 and *F. tricinctum* strain IBT 2952. The production of fusarielin was variable in different media containing different carbon and N sources. *Fusarium graminearum* IBT 9203 produced fusarielin H on a broad range of carbon sources, but produced a greater amount with nitrate as the N source (Figure 3, **IV**). The highest concentration of fusarielin H, 1.5 μg mL^{-1}, was detected on cellobiose with nitrate (Figure 3, **IV**). The second highest concentration of fusarielin H, 1.4 μg mL^{-1}, was produced on arabinose with arginine (Figure 3, **IV**). *Fusarium tricinctum* strain IBT 2952 produced the highest concentration of fusarielin A, 2.6 μg mL^{-1}, on dextrin (Figure 3, **IV**). This strain also produced a high quantity of fusarielin A on media with monosaccharides. The highest concentration of fusarielin A, 0.264 μg mL^{-1}, was obtained with arabinose, and the lowest concentration, 0.021 μg mL^{-1}, was obtained with fructose (Figure 3, **IV**). These data collectively indicated that fusarielin production is complex and it is influenced by N and carbon sources (**IV**).

5.4. Influence of abiotic factors (IV)

The influence of temperature, pH, time, and different concentrations of fructose on the production of fusarielin by *F. graminearum* strain IBT 9203 was studied. The fungus could not grow without fructose in the selected medium. Incubation temperature of 25 °C and pHs of 5

and 6 were the most suitable for fusarielin production (Figure 4, **IV**). The highest concentration of fusarielin was produced by fungus at 18 and 26 days after inoculation (Figure 4, **IV**). The highest concentration of fusarielin, 13.8 μg mL^{-1}, was at a fructose concentration of 60 mg mL^{-1} (Figure 4, **IV**). The highest concentration of fusarielin G, 13.4 μg mL^{-1}, and fusarielin F, 2.70 μg mL^{-1}, were also observed at the fructose concentration of 60 mg mL^{-1} (Figure 4, **IV**). When the concentration of the fructose increased to 120 and 180 mg mL^{-1}, then the production of fusarielin decreased 50% and 75%, respectively. At a fructose concentration of 240 mg mL^{-1}, fusarielin production was inhibited (Figure 4, **IV**). The highest concentration of fusarielin H, 17.5 μg mL^{-1}, occurred under slightly acidic pH conditions (pH = 6) (Figure 4, **IV**).

5.5. The effect of weather conditions (I, II, III)

The occurrence of *Fusarium* fungi in barley grains was variable in 2009 and 2010 in Central Estonia. In 2009, *Fusarium* spp. were found on 38% and in 2010, on 96% of grains in Central Estonia (**I**). The weather strongly differed among the two study years. In 2009, the sum of precipitation was 135.6 mm, average air temperature 17.1 ºC in July and 95.2 mm and 15.7 ºC in August. In 2010, the sum of precipitation was 42.8 mm and average air temperature 22.3 ºC in July and 143.2 and 18.6 ºC in August (Table 1, **I**).

The average abundance of moulds, yeasts and *Fusarium* spp. on grain was higher in 2010 than in 2009 ($p<0.05$; Figure 2a, **II**). The average air temperature was 3.3 ºC higher than the long-term average air temperature (14.4 ºC) in the plant growing season of 2010 in Central Estonia (**II**). The average air temperature was 2.1 ºC higher than the long-term average air temperature (13.8 ºC) also in North Estonia (**II**). Additionally, the average abundance of moulds was four times higher in Central Estonia compared to the abundance of moulds in North Estonia ($p<0.05$) (Figure 2b, **II**). In Central Estonia, the growing season sum of precipitation was higher, 20% in 2009 and 14% in 2010, compared to the long-term sum of precipitation (277 mm). In North Estonia, the growing season sum of precipitation, 74% in 2009 and 67% in 2010, was less compared to the long-term sum of precipitation (268 mm) (**II**).

The incidence of *Fusarium* spp. was 3.8% in 2012, 15.3% in 2013 and 10.9% in 2014 in barley grains (Table 3, **III**). The year-specific weather

conditions significantly affected the mean incidence of *Fusarium* spp. on barley grain ($p<0.05$) (Table 3, **III**). Likewise, the year affected the incidence of mycotoxins DON, H-T2 and T-2, indicating a significant weather effect ($p<0.001$) (Table 4, **III**). Both year and year*treatment significantly affected grain contamination by mycotoxin DON ($p<0.001$) (Table 4, **III**) and by HT-2 and T-2 ($p<0.001$) (Table 4, **III**). The weather conditions in 2012, 2013 and 2014 varied strongly in North Estonia. The average air temperature was 0.8 ºC lower in 2012, and 1.7 ºC and 0.7 ºC higher in 2013 and 2014 growing seasons compared to the long-term average air temperature (14.0 ºC) (Table 1, **III**). The sum of precipitation was 38% higher in 2012, and 6% and 12% less in 2013 and 2014 compared to the long-term sum of precipitation (269 mm) (Table 1, **III**). The average air temperature of July was 0.6 ºC, 0.8 ºC and 2.7 ºC higher in 2012, 2013 and 2014, respectively, than the long-term average air temperature (16.3 ºC) (Table 1, **III**). The average sum of precipitation in July was 42% higher in 2012, but 54% and 52% less in 2013 and 2014 compared to the long-term average sum of precipitation (90.0 mm) of July (Table 1, **III**). The average air temperature was 1.1 ºC lower, 1.0 ºC and 1.2 ºC higher in August in 2012, 2013 and 2014 compared to the long-term average (15.3 ºC) (Table 1, **III**). The average sum of precipitation was 41% and 51% higher in 2012 and 2013, but 6% lower in 2014 compared to the long-term average sum of precipitation (73.0 mm) (Table 1, **III**).

6. DISCUSSION

6.1. Effect of weather conditions (I, II, III)

Weather conditions had the greatest impact on the incidence of *Fusarium* spp. in 2009−2010 (**I**) and 2012−2014 (**III**) as well as on the abundance of moulds and yeasts in 2009−2010 (**II**) on barley grain. Several previous studies have confirmed that the weather is the main factor affecting the incidence of *Fusarium* spp. and other microfungi on cereal grain (Sutton, 1982; Torp & Nirenberg 2004; Uhlig *et al.,* 2007; Blandino et *al.,* 2008; Tirado *et al.,* 2010; Parikka *et al.,* 2012; West *et al.,* 2012). Parikka *et al.,* (2012), observed that warm air temperatures with heavy rainfall just before the harvest increased the spread of *Fusarium* spp., while high air temperature, drought, and intense rainfall caused plant stress, creating favourable conditions for *Fusarium* spp. infection in cereals in Northern Europe.

The incidence of mycotoxins DON, HT-2, and T-2 on barley grain varied over the study years (2012−2014) under the different weather condition (**III**). In Estonian climate, flowering, kernel development, and ripening of spring cereals occurs between July and the beginning of September. The duration of the growth stages depends on sowing time and weather conditions throughout the vegetation period (**III**). Other studies concur that the weather conditions, especially in the flowering stage, kernel development stage, and ripening stage, affect the incidence of *Fusarium* and mycotoxins on grain (Osborne & Stein 2007; Edwards, 2009a; Burlakoti *et al.,* 2011; Ferrigo, *et al.,* 2016; Mielniczuk & Skwaryło-Bednarz 2020). Heavy rainfall during these growth stages favours incidence of *Fusarium* spp. and mycotoxins on grain (Mankevičiene *et al.,* 2011). Malachova *et al.,* (2010) found that in several varieties of brewing barley, 86% of samples were contaminated with DON and 62% with HT-2. Nevertheless, in these studies, the largest factor affecting incidence of mycotoxins was weather (Malachova *et al.,* 2010). A four-year study with malting barley also concluded that the weather influenced the contamination of kernels with mycotoxins (Běláková *et al.,* 2014). In Estonia, the weather conditions during the growing season are highly variable among the years depending on the prevalence of low pressures from Atlantic or high pressures from continental Russia and southern Europe (Tammets & Jaagus 2013). Alhough the *Fusarium* incidence is

difficult to predict in a specific year due to the high weather variability, the data obtained provide a basis for risk assessment. Furthermore, given that global climate change is expected to result in wetter and variable climate in Northern Europe (Tammets & Jaagus 2013; Arnell *et al.*, 2019), the results of this dissertation suggest that *Fusarium* incidence is expected to further increase in the future.

6.2. Effect of ammonium nitrate and barley-pea intercropping (I, II)

The intercropping of barley (*Hordeum vulgare* L.) with field pea (*Pisum sativum* L.) decreased incidence of *Fusarium* spp. compared to the monotypic barley stand (**I**). This contrasts to other studies of cereal-legume intercropping where legumes have been used as cover crops or living mulches; these studies have shown an increase in the abundance of pathogenic *Fusarium* fungi in soil (Šišić *et al.,* 2018). Nevertheless, intercropping is a different treatment that actually reduces the share of cereal canopy relative to total canopy cover, and this could reduce the spread of *Fusarium* among cereal plants. The most frequent *Fusarium* spp. in all treatments was *F. avenaceum* (**I**). A similar result was found by Šišić *et al.,* (2018) though that study examined the leguminous roots.

Hofer *et al.* (2016) concluded that the incidence of *Fusarium* DNA in spring barley (*Hordeum vulgare* L.) did not differ between N treatments and natural field conditions. At the same time, the incidence of *F. avenaceum*, *F. culmorum,* and *F. langsethiae* DNA in barley grain decreased in all N fertilization treatments (Hofer *et al.,* 2016). Barbetti *et al.*, (2007) found that low soil organic matter content and nutrient-poor soils were favourable to *F. avenaceum*. Lv *et al.,* 2020 found that intercropping faba bean (*Vicia faba* L.) with wheat (*Triticum aestivum* L.) reduced *Fusarium oxysporum* in faba bean roots. In our study, *F. oxysporum* did not occur in the intercropping treatment but *Fusarium avenaceum, F. poae* and *F. sporotrichioides* occurred in treatments with the highest (N120) and lowest (N40) application rates of mineral fertilizer (**I**). In this study, the occurrence of *Fusarium* spp. was higher in the N40 and N120 treatments than in the N60 treatment (**I**). Also Yang *et al.*, (2010) found that barley was susceptible towards *Fusarium* spp. contamination in low nitrogen level. Studies with winter wheat (*Triticum aestivum* L.) (Hemissi *et al.,* 2018; Vrandečić *et al.,* 2019) and oat (*Avena sativa* L.) (Guo *et al.,* 2018) showed that high N rates, N115 (Vrandečić *et al.,* 2019), N150 (Guo *et*

al., 2018; Hemissi *et al.,* 2018; Vrandečić *et al.,* 2019), and N200 (Hemissi *et al.,* 2018) increased *Fusarium* spp. infections on the grain (Hemissi *et al.,* 2018; Vrandečić *et al.,* 2019) and roots (Hemissi *et al.,* 2018). N application influences the chemical composition and cell wall structure of the plants which may make them more susceptible to mould attack (van Arendonk *et al.,* 1997). On the other hand, N availability also alters soil microbial community composition and activity (Geisseler *et al*., 2010; Chen *et al*., 2018) that can further affect *Fusarium* incidence (Marshall & Alexander 1960; Gu *et al*., 2020). Thus, study-to-study differences in N and intercropping effects on *Fusarium* occurrence might be associated with differences in initial soil conditions and magnitude of changes in soil and plant characteristics.

Moulds *Cladosporium* spp., *Acremonium* pp., and *Fusarium* spp. are the most common saprophytes on cereals; they appear in the soil and on the plants (Domsh *et al.,* 2007, Vujanovic *et al.,* 2012). Georgieva *et al*., (2005) concluded that amount of fungal biomass during decomposition of plant material depends on the plant species For example; rye residues were inhabited by 89% of fungal hyphae, but only 18–31% of wikis residues (Georgieva *et al*., 2005). The moulds feed on the organic matter in the soil (Madigan *et al*., 2003). The hydrolytic enzymes of moulds degrade starch, lignin, and cellulose into simpler substances which are absorbed by hyphal cells (Madigan *et al*., 2003; Kikot *et al*., 2009). Moulds are major decomposers of organic material, enabling recycling of nutrients throughout the ecosystem (Madigan *et al.,* 2003). Adding a large amount of fresh plant biomass into the soil, e.g. as manure or green manure, increases the abundance of *Fusarium* fungi as the fungi quickly start to decompose the plant biomass (Kikot *et al*., 2009; Leplat *et al*., 2012). The abundance of moulds, yeasts, and *Fusarium* spp. on barley grain in two locations (Central and North Estonia) differed greatly by the microbial groups, N fertilization, and weather conditions (**II**). The incidence of yeasts on barley grain was higher compared to the incidence of moulds and *Fusarium* spp. (**II**). Olstorpe *et al.,* (2010) also found that the microbial flora on barley grain varied considerably. High N application increased the severity of infestation by obligate pathogenic fungi, such as *Puccinia* spp.; it may decrease the infestation of facultative pathogenic fungi, such as *Alternaria* spp. and *Fusarium* spp. (Dordas, 2008). The abundance of *Fusarium* spp. was low (1.6%) compared with the abundance of other moulds (**II**). *Fusarium* spp. strains are rare compared to the common species of moulds and yeasts (Krysinska-

Traczyk *et al.,* 2007). Some yeasts and moulds, for example, the genera *Acremonium* spp., inhibit the growth and progression of *Fusarium* spp. on grain which may be the reason why the incidence of *Fusarium* spp. strains are relatively rare (Vujanovic *et al.,* 2012). This evidence further emphasizes that soil biological factor can importantly drive the effects of soil N addition treatments on *Fusarium* spp. occurrence as discussed in the section 6.2.

6.3. Effect of fungicides (III)

The effect of fungicides varied over the three study years; only the fungicide with active ingredients fenpropidin with propiconazole decreased the incidence of *Fusarium* spp. on barley grain (**III**). In Lithuanian field trials, a mixture of prothioconazole and tebuconazole effectively decreased *Fusarium* spp. contamination on barley grain (Semaškiene *et al.,* 2006). Semaškiene *et al.* (2006) also found that weather had a strong impact on the efficacy of fungicides on *Fusarium* spp. Contrary to this study and Semaškiene *et al.* (2006), several other studies have demonstrated that single fungicides, e.g. tebuconazole, are effective in controlling *Fusarium* incidence (Suproniene *et al*., 2011a; Sun *et al*., 2014). Fungicides with various active ingredients may support the production of trichothecenes by *Fusarium* spp. in wheat (*Triticum aestivum* L.) (Mateo *et al.,* 2011, Giraud *et al.,* 2011), in rye (*Secale cereale* L.), and in triticale (*xTriticosecale* Wittm.) kernels (Gaurilčikienė *et al.,* 2011).

The incidence of mycotoxins DON, HT-2, and T-2 in barley kernels varied strongly over the three years of study (**III**). DON was detected in all years but it only occurred in the untreated control and tebuconazole treatment in 2014. The DON concentrations in all cases were below the maximum allowed limit (1250 μg kg^{-1} according to European Commission legislation; EC 1881/2006) (**III**). HT-2 and T-2 were found in all treatments in 2012, but only in the untreated control and tebuconazole treatment in 2014. Thus, the use of fungicides to prevent the formation of mycotoxins in barley grains did not achieve the desired results (**III**). In France, winter barley in naturally infected conditions had a very low average DON content (<20 μg kg^{-1}) over three experimental years, while fungicide treatment had a no clear effect on the *Fusarium* spp. infection rate (Ioos *et al.,* 2005). In the first experimental year better results were achieved using a complex fungicide with a mixture of active ingredients (Ioos *et al.,* 2005). In the second year, the six single-ingredient fungicides

more strongly suppressed *Fusarium* spp. than the mixed fungicide, and in the third experimental year only one fungicide was effective against *Fusarium* spp. (Ioos *et al.,* 2005). Moreover, the fungicide treatments had no effect on the production of DON and nivalenol (Ioos *et al.,* 2005). The results of our experiment are in agreement with the study of Ioos *et al.* (2005) in that the applied fungicides were not effective against contamination by mycotoxins DON, HT-2, or T-2 (**III**).

6.4. Impact of nitrogen and carbon sources (IV)

Two *Fusarium* species of the fifteen species tested, *F. graminearum* and *F. tricinctum*, produced different fusarielin-type mycotoxins (**IV**). Sørensen *et al.,* (2012) found that *F. graminearum* strains produced mainly fusarielin H and smaller amounts of fusarielin G and F; the production of fusarielins was higher in YES media compared to other media. *Fusarium culmorum* and *F. pseudograminearum*, the two species very closely related to *F. graminearum*, did not produce fusarielins (**IV**). These fusarielin-non-producing are genetically very similar to *F. oxysporum* and *F. verticillioides*, where the analysis of the whole genome sequence revealed the absence of the fusarielin gene cluster (Hansen *et al*., 2012). Similarly, *F. tricinctum* is genetically related to *F. avenaceum* (Yli-Mattila *et al*., 2002) alone produced fusarielin-type mycotoxins (**IV**), although both species produce the secondary metabolites for example enniatins and moniliformin (Langseth *et al*., 1999).

Carbon and N sources have an effect on the magnitude of production of mycotoxins in *Fusarium* spp. This study demonstrates that the regulation of fusarielin production is complex and is strongly influenced by N and carbon sources (**IV**). Other secondary metabolites are influenced by the N source; *F. graminearum* with arginine as the N source produced more DON than it did with nitrate as the N source and sucrose as the carbon (Gardiner *et al*., 2009). Gardiner *et al*., (2009) tested 30 nitrogen sources and showed that nitrate as the N source had a stronger repressive effect on DON production than any of the tested different nitrogen sources (for example compared arginine, agmatine, putrescine). However, Jiao *et al*., (2008) found that carbon sources also influenced DON production. The N and carbon sources both influenced the production of the red pigment aurofusarin by *F. graminearum* (Zhang & Wolf-Hall 2010). These results confirm that the N regulator, transcription factor AreA and, the carbon regulator, transcription factor creA, have a strong regulatory

influence on the biosynthesis of secondary metabolites in filamentous fungi (Yu & Keller 2005).

The high fructose content (240 mg mL^{-1}) caused an osmotic stress, and thereby led to an inhibitory effect on fungal growth and mycotoxin production (Ben Gaida *et al.*, 2006). Osmotic conditions influenced the production of zearalenone and DON in *F. graminearum* (Hope *et al.*, 2005; Kokkonen *et al.*, 2010). Mycotoxins fumonisins were also affected by changes in osmotic conditions induced by glucose, sodium chloride (NaCl), and glycerol (Mogensen *et al.*, 2009). This dissertation showed that fusarielins are weakly influenced by medium pH, whereas pH 5–6 was optimal for fungal development (**IV**). In previously study showed also that weakly acid condition favoured production of zearalenone (Kim *et al.*, 2005). The transcription factor PacC regulates secondary metabolites in acidic and basic conditions (Tilburn *et al.*, 1995), including DON in *F. graminearum* (Merhej *et al.*, 2011) and bikaverin in *F. fujikuroi* (Wiemann *et al.*, 2009). The results of this thesis and literature evidence together indicate that at the given *Fusarium* infection level, the composition of formed mycotoxins is driven by the complex of *Fusarium* species supported by the host plants. In addition to the infection severity, the amount of specific mycotoxins produced can significantly depend on environmental conditions (temperature and humidity) and host plant quality, i.e. surface pH, and availability of different N and carbon sources.

CONCLUSIONS

- Barley–pea intercropping reduced the incidence of *Fusarium* spp. and other microfungi on barley grains compared to barley grown with mineral N fertilizer (**I**). The most frequently occurring species in all mineral N fertilizer treatments and in the barley–pea intercropping treatments was *Fusarium avenaceum*.

- Fertilization with mineral N had a limited (**I**) or no effect (**II**) on the total incidence of mould, yeasts, or *Fusarium* spp. on barley grain. Study site location and year influenced incidence of moulds and yeasts, but not the incidence of *Fusarium* spp.

- Results from three-year field trials demonstrated that the incidence of *Fusarium* fungi and mycotoxins on grains varied among years (**III**). The fungicides used to prevent *Fusarium* fungal development and mycotoxin production also varied from year to year. The results suggest that fungicide commercial mix fenpropidin and propiconazole decreased the incidence of *Fusarium* fungi on grain. The use of fungicides did not prevent the presence of mycotoxins DON, H-T2, or T-2 on spring barley grain (**III**).

- Of the 15 *Fusarium* species tested the ability to produce fusarielins was restricted to *F. graminearum* and *F. tricinctum*. The production of fusarielins was influenced by ambient conditions including N and carbon sources, time, temperature, and pH (**IV**).

In this thesis, the contamination of cereals with *Fusarium* fungi was compared among the treatments with different nitrogen (N) source, cereal and legume intercropping (as a biological nitrogen (N) source) vs. fertilization with a mineral N fertilizer (Paper I). Furthermore, the impacts of intercropping vs. monotypic stands on the microbiological quality of the harvest were studied in different field locations and in dependence on weather conditions (Paper II). The dissertation analysed the success of the recommended *Fusarium* control methods by the use of fungicides during flowering (Paper III) and the formation of mycotoxins in the grains in different climatic conditions (Papers III). Finally, the effect of sourses of carbon and nitrogen, acidity of environment, temperature and time on ability mycotoxin production

of *Fusarium* species explained (IV). The results of this dissertation collectively provide new knowledge of the effects of cereal and legume intercropping, cultivation technologies, weather and *Fusarium* control strategies on the contamination of cereals with *Fusarium* fungi. The dissertation demonstrated that use of a biological N source in the legume intercropping system improved grain microbiological quality. The dissertation also showed that field location and weather significantly contributed to the microbiological quality of the harvest. In research appear the conditions of environment, also the structure carbon (C) and nitrogen (N) affect the mycotoxin production on *Fusarium* regulatory genes. Analysis of the recommended *Fusarium* control methods indicated that the success of curbing *Fusarium* infestation spread and presence of mycotoxins in the grains is fungicide independent and highly variable among the years due to differences in weather, implying that precisely tailored approaches are needed for northern humid climates. The results of this dissertation collectively provide new knowledge of the effects of cereal and legume intercropping and cultivation technologies on the contamination of cereals with *Fusarium* fungi.

Future research:

These results have highlighted multiple intriguing tendencies of how weather and intrinsic plant factors can alter *Fusarium* occurrence and mycotoxin formation, but long-term research is needed to reach at definitive conclusions. Other mycotoxins which also pose a risk to human and animal health should be analysed in addition to the ones in this study, DON, T-2, and HT-2. It is important to better understand the effect of weather and growing technologies on incidence of *Fusarium* fungi in order to predict *Fusarium* fungi and mycotoxin contamination risks during each plant growth phase.

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SUMMARY IN ESTONIAN

Teravili on kogu maailmas oluline kaubaartikkel, mida kasvatatakse nii inimtoiduks kui ka loomasöödaks. Eesti teravilja eksport 2010. aastal oli 35 miljonit eurot, 2016. aastaks kasvas see 107 miljoni euroni (Eesti Majandus, 2017). Teravilja kvaliteeti hinnatakse füüsikaliste näitajate (niiskusesisaldus, mahukaal, terade suurus ja mass, kahjustatud terad), koostise (valgu- ja tärklisesisaldus, jahvatamise saagis, elujõulisus) ja ohutuse näitajate alusel (putukad, lestad, seeninfektsioon, mükotoksiinid, tolm ja lõhn) (Jayas & Singh, 2012). Teravilja käitlevad tööstusettevõtted ei osta mikroseentega ja mükotoksiinidega saastunud toiduteravilja (Varzakas, 2016). Söödavilja puhul on lubatud *Fusarium* seentega nakatunud terade sisaldus 0,1%, kuid mitte selle toksilisus (Scandagra, 2018; Tartumill, 2018; Balticagro, 2018).

Maailmas on erinevaid *Fusarium* seeni nii mullas kui teraviljadel väga palju uuritud (Nirenberg, 1989; Doohan *et al.,* 2003; Koch *et al.,* 2006; Medina *et al.,* 2006; Richard, 2007; Fernandez *et al.,* 2008; Chakraborty & Newton, 2011; Parikka *et al.,* 2012; West *et al.,* 2012; Yli-Mattila *et al.,* 2011, 2013; Hietaniemi *et al.,* 2016). Neist uurimustest on selgunud, et *Fusarium* seente arvukusele mullas ja teradel avaldavad olulist mõju agronoomilised võtted nagu külvikord, harimistehnoloogia, viljelussüsteem, väetamine, pestitsiidide kasutamine, aga samuti teravilja liik ja sort ning ilmastikutingimused (Koch *et al.,* 2006; Fernandez *et al.,* 2008; Schaafsma & Hooker 2007; Shwab & Keller 2008; Bernhoft *et al.,* 2012; Parikka *et al.,* 2012; West *et al.,* 2012). Erinevad agrotehnilised võtted ja ilmastikutingimused mõjutavad ka teiste patogeensete mikroseente levimist põllul (Doohan *et al.,* 2003; Parikka *et al.,* 2012), samuti mikroorganismide arvukust ja rohkust mullas (Glick, 1995). On leitud, et *Fusarium* seente esinemine on madal kõrge mikrobioloogilise aktiivsusega muldadel (Perez *et al.,* 2008; Yogev *et al*., 2011).

Fusarium seente uurimist alustati juba 19. sajandi alguses (Link, 1809). Tänaseks leidub Mycobank andmebaasis ligi 1700 nimetust erinevaid *Fusarium* seeni (Mycobank, 2020). Seeneperekond *Fusarium* on maailma muldades laialt levinud, kuna nad asustavad püsivalt saprotroofselt taimejäänuseid (Sutton, 1982; Pereyra & Dill Mackey 2008; Fernandez *et al*., 2008) ja lagundavad mullas orgaanilist ainet (Sutton, 1982; Kosiak *et al*., 2004; Popovski & Celar 2013).

Teraviljadel võib tugev *Fusarium* seentega nakatumine põhjustada terade kvaliteedi halvenemist, saagikuse langust (Medina *et al*., 2006; Richard, 2007; Chakraborty & Newton 2011) ja mükotoksiinidega saastumist (Richard, 2007). *Fusarium* nakkus levib teradesse peamiselt teraviljade õitsemise ajal, kui seente eosed kinnituvad õietolmule (Tekle *et al*., 2012; Persson *et al*., 2017). Soodsad keskkonnatingimused nakatumiseks on õhutemperatuuri vahemik 10–25 ºC ning kõrge õhuniiskus, mis ei lase taimedel 24 tunni vältel kuivada (Rossi *et al*., 2001).

Mükotoksiinideks nimetatakse mikroseente poolt toodetud sekundaarseid metaboliite (seente ainevahetuse tulemusel tekkinud ühendid), mis toidu-ja söödaviljas põhjustavad mürgitusi nii inimestele (Bryden, 2007) kui loomadele (Pinotti *et al*., 2016). *Fusarium* seened võivad toota ühte või mitut erineva mürgisuse tasemega mükotoksiini (Bottalico & Perrone 2002). Seente poolt toodetud looduslikke ühendeid on avastatud üle 170 000 (Gruber-Dorninger *et al*., 2017), neist mükotoksiine teatakse üle 300 keemilise kombinatsiooni (Alshannaq & Yu 2017). Mükotoksiinid jagunevad järgmistesse gruppidesse: trihhotetseenid, aflatoksiinid, ohratoksiinid, fumonisiinid, zearalenoon, patuliin, ergot alkaloidid, alternarioolid ja paljud teised (Gruber-Dorninger *et al.,* 2017). Teraviljades ja nendest valmistatud toodetes lubatud maksimaalsed mükotoksiinide piirmäärad kehtestati Euroopa Komisjoni Regulatsioonis No 1881/2006 (Euroopa Komisjon, 2006). Soovitatud maksimaalsed piirmäärad mükotoksiinidele HT2 ja T2 kehtestati 2013. aastal (EU 165/2013).

Käesolevas väitekirjas esitatakse Eestis ja Taanis läbi viidud teadustöö publitseeritud tulemused. Eestis tehtud uurimistöö üldeesmärgideks oli välja selgitada teravilja ja kaunvilja segukülvi mõju teravilja *Fusarium* seentega nakatumisele. Samuti võrreldi segukülvi kui lämmastiku allika ning kasvuajal lisatud mineraalse lämmastiku mõju saagi saastumisele *Fusarium* seentega. Uuriti ka põllu asukoha ja ilmastiku mõju saagi mikrobioloogilisele kooslusele. Vajalik oli välja selgitada, kas teravilja õitsemise aegne fungitsiididega pritsimine võimaldab vähendada või ära hoida saagi *Fusarium* seentega nakatumise ja mükotoksiinide tekkimise. Taanis Aarhusi Ülikoolis läbiviidud laborikatsetes uuriti millised keskkonna tingimused mõjutavad erinevate *Fusarium* liikide mükotoksiinide tootmise võimet.

Täpsemalt olid käesoleva töö eesmärgid järgmised: 1) analüüsida *Fusarium* seente liigilist koostist odra teradel mineraalse lämmastikväetisega

väetamisel ja herne-odra segukülvis kasvatamisel (**I**); 2) uurida puhaskülvis ja segukülvis liblikõielisega kasvatatud teravilja saagi hallitus- ja pärmseente ning *Fusarium* seente arvukust teradel erinevatel aastatel ja asukohtades (**II**); 3) selgitada teravilja õitsemis-aegse fungitsiididega pritsimise mõju *Fusarium* spp. ja mükotoksiinide DON ja HT-2+T-2 tekkimisele terasaagis (**III**); 4) uurida, millistes keskkonnatingimustes toimub mükotoksiinide tootmine (**IV**).

Doktoritöös püstitati järgmised hüpoteesid:

1) odra kasvatamisel segus põldhernega väheneb *Fusarium* spp. seente esinemine teradel võrreldes mineraalsel lämmastiku foonil kasvatatud odraga (**I**);
2) ilmastik, põllu asukoht, väetamisega antud lämmastiku kogus ja suviodra põldhernega segus kasvatamine mõjutavad hallitus-, pärmseente ja *Fusarium* seenet arvukust teradel erinevalt (**II**);
3) suviodra fungitsiididega õitsemisaegne pritsimine vähendab *Fusarium* spp. esinemist ja mükotoksiinide DON, HT-2 ja T-2 tekkimist odrasaagis (**III**);
4) *Fusarium* spp. mükotoksiinide tootmine sõltub seente kasvuks saadaolevatest süsiniku ja lämmastiku struktuurist ning mükotoksiinide tootmist mõjutavad keskkonnatingimused ja aeg (**IV**).

Uurimustöö läbiviimiseks rajati järgmised põldkatsed:

1) Aastatel 2009–2010 (**I, II**) Kesk-Eestis Olustveres (58º33´N, 25º34´E), leetunud mullal *Podzoluvisol* (FAO, ISSS, ISRIC. 1998).

2) Aastatel 2009–2010 (**II**) Põhja-Eestis (koordinaadid) rähkmullal, *Cambisol* (FAO, ISSS, ISRIC. 1998).

3) Aastatel 2012–2014 Põhja-Eestis (59º18´N, 25º39´E) kamar-gleimullal, *Gleysol* (FAO, ISSS, ISRIC 1998). (**III**).

Katsete rajamisele eelneval sügisel alad künti. Kahes põldkatses kasvatati suviodra (*Hordeum vulgare* L.) sorti 'Anni' ja herne (*Pisum sativum* L.) sorti 'Clarissa' (**I, II**) ja ühes katses suviodra sorti 'Maali' (**III**). Kahe põldkatse variandid olid järgmised: 1) suvioder lämmastiku fooniga 120 kg ha^{-1}; 2) suvioder lämmastiku fooniga 60 kg ha^{-1}; 3) suvioder lämmastiku fooniga 40 kg ha^{-1}; 4) suviodra-herne segukülv. Kõikidele variantidele anti lämmastik

40 kg ha $^{-1}$ koos odraseemne külviga, kõrgema lämmastikunormiga variante väetati lisaks ammooniumnitraadiga taimede kõrsumise kasvufaasi alguses. Umbrohutõrje teostati herbitsiidiga Butoxone (toimeaine MCPB) (**I**, **II**). Kolmanda, fungitsiididega katse variandid olid: 1) pritsimata kontroll, 2) pritsitud toimeainega tebukonasool, 3) pritsitud toimeainete protiokonasool, spiroksamiin ja tebukonasool seguga, 4) pritsitud toimeainete fenpropidiin ja propikonasool seguga. Fungitsiidega pritsiti otra õitsemise kasvufaasis. Umbrohutõrje tehti herbitsiidiga MCPA (toimeaine 2-metüül-4-klorofenoksüäädikhape) (**III**). Saak koristati kombainiga, kuivatati 14% niiskuseni, puhastati ning igast variandist võeti 1,5 kg proov (**I, II, III**), milledest tehti *Fusarium* seente (**I, II, III**) ja mükotoksiinide analüüsid (**III**).

Laboratoorsed katsed fusareliinide määramiseks viidi läbi Taanis Århusi Ülikoolis (**IV**). Katsesse valiti viisteist *Fusarium* liigi isolaati (*F. avenaceum*, *F. cerealis*, *F. culmorum*, *F. graminearum*, *F. equisety*, *F. langsethiae*, *F. oxysporum*, *F. poae*, *F. proliferatum*, *F. pseudograminearum*, *F. sambucinum*, *F. solani*, *F. sporotrichioides*, *F. tricinctum*, *F. venenatum*), mis saadi Taani Tehnikaülikooli kollektsioonist ja Põllumajanduse Uurimisteenistuse USA Illinoisi Põllumajanduse Uuringute Riikliku Keskuse kollektsioonist. Laboratoorses katses hinnati *Fusarium* seente võimet toota mükotoksiini fusareliin (**IV**). Fusareliinide A, F, G ja H kvantitatiivne määramine viidi läbi Dionex UltiMate 3000 UHPLC kromatograafiga. Selleks, et saavutada võimalikult palju *Fusarium* seente reageerimist ja suurendada geenide aktiivsust, kasvatati isolaate viiel erineval söötmel. Keskkonna tingimuste mõju fusareliinide tekkimisele uuriti *Fusarium graminearum* isolaadiga (**IV**).

2009. aastal jagus taimedele soodsaid õhutemperatuure nii Kesk- (tabel 1, **I**) kui Põhja-Eestis (**II**), kuid sademete jaotus oli ajaliselt ja ruumiliselt ebaühtlane. 2010. aastal olid taimede kasvuaegsed keskmised õhutemperatuurid Põhja- Eestis 2,1 ºC (norm 13,8 ºC) ja Kesk-Eestis 3,3 ºC (14,4 ºC) võrra kõrgemad pikaajalisest keskmisest õhutemperatuurist. 2009. aastal esines taimede kasvuajal sademeid Põhja-Eestis 74% ja Kesk-Eestis 120% normist (normid vastavalt 268 mm ja 277 mm), 2010. aastal vastavalt 67% ja 114% normist. Kesk-Eestis sadas 2009. aastal juunis ja juulis rohkelt (vastavalt 85,3 mm ja 135,6 mm), samal ajal valitses Põhja-Eesti katsekohas põud ja normaalsel hulgal sademeid tuli alles augustis. 2010. aastal sadas Kesk-Eestis juulikuus vähe (sademete summa 42,8 mm) ning augustis rohkelt (sademete summa 143,2 mm) (**I, II**).

Katsete tulemusel tuvastati suviodra terades 12 mikroseene liiki. Domineeris liik *Fusarium avenaceum* (**I**), mida esines kõikides katsevariantides (**I**). *Fusarium* seente esinemine erines 2009. ja 2010. aastal Kesk-Eestis kasvanud odras oluliselt (tabel 2, **I**). 2009. aastal esines *Fusarium* seeni keskmiselt 38% odra teradest, 2010. aastal 96% (**I**). Katseaasta ilmastik mõjutas oluliselt *Fusarium* seente esinemist ($p<0,05$) (**I**), samuti avaldasid tuvastatud mikroseente keskmisele esinemise sagedusele odra terades mõju lämmastiku määrad ja odra kasvatamine segus hernega (joonis 1, **I**). Lämmastiku N120 ja N40 foonil esines mikroseeni terades oluliselt rohkem kui N60 ja odra segukülvis hernega ($p<0,05$) (joonis 1, **I**). Odra teradel kasvasid kõige sagedamini liigid *Fusarium avenaceum* ja *Fusarium poae* (tabel 2, **I**). Odra-herne segukülvist pärit odra terades tuvastati vaid üks *Fusarium* liik - *Fusarium avenaceum* ($p<0,05$) (tabel 3, **I**). Katse tulemused näitasid, et odra kasvatamine segus hernega on üks oluline kasvatustehnoloogiline võte odra terade nakatumise vähendamiseks *Fusarium* seentega (**I**). Ammoniumnitraadiga kasvuaegsel pealtväetamise variantides (N120 ja N60) ja ilma pealtväetamiseta variandis (N40) esinesid odra terades lisaks *Fusarium avenaceum*'ile ka liigid *Fusarium poae* ja *Fusarium sporotrichioides* (**I**).

Uurimistöös selgus, et mikrobioloogiline kooslus suviodra teradel oli väga erinev (joonis 1, **II**). Hallitus-, pärmseente ja *Fusarium* seente arvukus oli kõrgem 2010. aasta teraproovides võrreldes 2009. aasta proovidega ($p<0,05$) (joonis 2a, **II**). 2009. aastal domineerisid teradel peamiselt hallitusseened, 2010. aastal pigem pärmseened (joonis 2a, **II**). Katseaasta mõju hallitus-, pärm ja *Fusarium* seente arvukusele oli statistiliselt usutav ($p<0,05$). Võrreldes hallitusseente ja pärmseente arvukusega jäi *Fusarium* seente arvukus odra teradel väga madalaks, moodustades vaid 1,6% määratud mikroseente suhtest (joonis 1, **II**). Kesk-Eesti põllult pärit odrateradel oli hallitusseente osakaal Põhja-Eesti katsepõllult pärit odraga võrreldes neli korda ja pärmseente osakaal kaks korda kõrgem ($p<0,05$) (joonis 2b, **II**). Seega oli erinevatest põllu asukohtadest pärit oder erineva mikrobioloogilise kooslusega. Samas ei avaldanud mineraalse lämmastikuga väetamine ja odra herne segukülvis kasvatamine kummaski asukohas mõju odra teradel paiknevate hallitus-, pärm ja *Fusarium* seente arvukusele ($p>0,05$) (joonis 2c, **II**).

Katseaastatel 2012–2014 oli taimede kasvuaegne ilm väga erinev (tabel 1, **III)**. Võrreldes pikaajalise sademete keskmisega oli 2012. aastal ilm pigem märg ja jahe. 2013. aastal olid ilmaolud kuumad ja kuivad, kuid augustis,

kui oder küpses, sadas palju. 2014. aastal varieerusid õhutemperatuurid ja sademete hulk igas kuus. Juuni oli jahe ja sademeterohke, juuli kuum ja kuiv, august kuum ja sademeid normi piires (table 1, **III**).

Kasvuaasta mõjutas oluliselt *Fusarium* seente esinemist terades (**III**). 2012. aastal oli *Fusarium* seentega nakatunud keskmiselt 3,8% teradest, 2013. aastal 15,3% ja 2014. aastal 14,0% (tabel 3, **III**). Fungitsiidide mõju terade *Fusarium* seentega nakatumisele avaldus kolme aasta keskmisena (p>0,05) (tabel 3, **III**). Vähem olid terad nakatunud pritsimata kontrollvariandis (7,7%) ja fungitsiidi toimeainete fenpropidiini ja propikonasooli seguga (4,0%) variandis (p<0,05) (table 3, **III**).

Uurimistöö tulemused tõid esile tugeva seose katseaasta ilmastiku ja mükotoksiinide tekkimise vahel, samuti aasta ja fungitsiidi kasutamise koosmõju olulisuse (tabel 4, **III**). Vihmased ilmad ja madalad temperatuurid (tabel 1, **III**) 2012. aasta kasvuperioodil soodustasid mükotoksiinide DON ja HT-2+T-2 tekkimist teradesse (tabel 4, **III**), ja seda kõikides katsevariantides. Seejuures mükotoksiini DON sisaldus terades oli kõrgem (p=0,001) fungitsiidiga töötlemata variandis (73,3 µg kg^{-1}) ning mükotoksiin HT-2+T-2 sisaldus oli kõrgeim (p=0,001) fungitsiidi toimeainete fenpropidiini ja propikonasooli seguga pritsitud variandis (62,3 µg kg^{-1}). 2013. aastal, mil kasvuperiood oli üldjoontes kuiv ja soe, kuid vihma sadas odra valmimise ajal (tabel 1, **III**), saastus oder kõikides katsevariantides mükotoksiiniga DON. Toksiini sisaldus oli veidi kõrgem fungitsiididega pritsitud variantide terades (63,1 µg kg^{-1}), võrreldes pritsimata variandi teradega (63,0 µg kg^{-1}) (p=0,009) (tabel 4, **III**). 2014. aasta kasvuaegne ilm oli soojem ja kuivem võrreldes 2012. ja 2013. aasta sama perioodiga (tabel 1, **III**). 2014. aastal tekkisisd mükotoksiinid DON ja HT-2+T-2 fungitsiidiga pritsimata (DON 65,5 µg kg^{-1}; HT2+T2 32,9 µg kg^{-1}) ja tebukonasooliga (DON 64,8 µg kg^{-1}; HT2+T2 32,6 µg kg^{-1}) pritsitud odra teradesse (P<0,001) (**III**).

Laboris viiel erineval söötmel katsetati 15 *Fusarium* seene liigiga mükotoksiinide fusareliinide tootmise võimet. Selgus, et fusareliini A ja H tootis ainult liik *F. tricinctum* ja fusareliine F, G ja H liik *F. graminearum* (tabel 2, **IV**). Fusareliinide tootmine erinevates *F. tricinctum* tüvedes varieerus oluliselt sõltudes substraadist ja selle kogusest. Kaks *F. tricinctum* tüve tootsid suurima koguse fusareliini A kahel erineval söötmel (CZ ja YES). Kolmas tüvi tootis madalama koguse fusareliini H, CZ söötmel, kuid kõrgeima koguse YES söötmel. Madalaim kogus fusareliini H toodeti

kõikide *F. tricinctum* tüvede puhul MEA söötmel (tabel 2, **IV**). Kolm liigi *F. graminearum* tüve tootsid kõrgeima fusareliinide koguse YES söötmel võrreldes teiste söötmetega (table 2, **IV**). Uuriti ka 18 erineva süsiniku vormi mõju *F. graminearumi* ja *F. tricinctumi* tüvede (vastavalt IBT 9203 ja IBT 2952) fusareliinide tootmise võimele (**IV**). Uuringust selgus, et polüsahhariide kasutades tootis *F. tricinctum* kõige rohkem fusareliini A. Polüsahhariidil dekstriin kasvamisel tootis seen kõrgeima fusarieliinide kontsentratsiooni, 2,6 μg mL^{-1} (joonis 3, **IV**). Kõrge koguse fusareliine A tootis *F. tricinctum* kasutades polüsahhariide maltoos, sahharoos, trehhaloos ja ksülaan. *F. graminearum* puhul mõjutas fusareliinide tootmist nii lämmastiku kui ka süsiniku vorm (joonis 3, **IV**). Lämmastiku vormi nitraadi kasutamisel toodeti seene poolt fusareliini H laiemast süsiniku vormide valikust. Nitraadi kasutamisel saadi fusareliin H kõige kõrgem produktsioon disahhariidil tsellobioosil kasvanud kultuurides (1,5 μg mL^{-1}), millele järgnesid fruktoos (monosahhariid), arabinoos (monosahhariid) ja maltoos (disahhariid). Kui lämmastikuallikana kasutati arginiini, saadi fusareliini H kõige suuremal määral arabinoosil (1,4 μg mL^{-1}), millele järgnesid laktoos (oligosahhariid), tsellobioos (disahhariid) ja trehhaloos (disahhariid). Fusareliini H toodeti koos nitraadiga kõigil monosahhariididel, kuid arginiini kasutamisel ainult arabinoosil. Tulemustest järeldus, et *Fusarium* seene mükotoksiin fusareliinide biosünteesi reguleerisid süsiniku ja lämmastiku vormid (**IV**).

F. graminearum tüvega, IBT 9203 uuriti keskkonna faktorite mõju (aeg, keskkonna happesu, temperatuur, fruktoosi kontsentratsioon) mükotoksiin fusareliinide tootmisele. Katses selgus, et fusareliinide F, G ja H tootmine suurenes koos ajaga, fusareliinide G, F ja H maksimaalsed kogused määrati vastavalt 18 ja 26 päeval (joonis 4, **IV**). Seen tootis suurima koguse fusareliine nõrgalt happelises kuni neutraalses keskkonnas (pH 5–6) (**IV**). Kõige optimaalsem keskkonna temperatuur fusareliinide tootmiseks oli 25 ºC, ja oli 88 ja 86% võrra madalam vastavalt 20 ºC ja 30 ºC temperatuuridel (joonis 4, **IV**). Seen *F. graminearum* ei suutnud kasvada ilma fruktoosita ja kõrgeima kontsentratsiooniga fusareliinide sisaldus analüüsiti fruktoosi kontsentratsioonil 60 mg mL^{-1}. Suure fruktoosi kontsentratsiooniga (240 mg mL^{-1}) söötme peal kasvas seen küll hästi, aga täielikult pärsitud oli fusareliinide tootmine (joonis 4, **IV**).

Seega mükotoksiin fusareliinide tootmist mõjutasid mitmed keskkonna tingimused, nii süsiniku ja lämmastiku vorm, aeg, keskkonna happesus ja temperatuur (**IV**).

Põhijäreldused on järgnevad:

- Võrreldes odra kasvatamisel mineraalse N väetise kasutamisega siis odra-herne segukülvi kasvatustehnoloogia vähendas saagis teradel *Fusarium* seente esinemist (**I**). *Fusarium* seeni esines sagedamini ammooniumnitraadiga pealtväetatud (N120 ja N60) ning ainult külviga lämmastikku saanud (N40) variantides. Suviodra teradel domineerisid liigid *Fusarium avenaceum* ja *F. poae* (**I**).

- Oluliselt mõjutas üldist hallitus-, pärmseente ja *Fusarium* seente arvukust odra teradel põllu asukoht (**II**). Hallitus- ja pärmseente seente arvukus oli kordades kõrgem Kesk-Eestist kasvanud odral (**II**). *Fusarium* seente arvukus oli 1,6% hallitus-ja pärmseente arvukuse suhtest (**II**).

- Ammooniumnitraadiga lämmastikuga pealväetamine ja odra-herne segukülvi kasvatustehnoloogia ei mõjutanud üldist hallitus-, pärmseente ja *Fusarium* seente arvukust ei Põhja- ega Kesk-Eestis kasvanud odras (**II**).

- Uuringu tulemused näitasid, et *Fusarium* seente ja mükotoksiinide esinemine teradel varieerus aastate lõikes. Samuti varieerus aastate lõikes fungitsiidide toime *Fusarium* seente esinemisele ja mükotoksiinide vältimiseks. Tulemustes järeldus, et toimeainete fenpropidiin ja propikonasooli segu vähendas *Fusarium* seente esinemist teradel. Fungitsiidide kasutamine odra õitsemise ajal ei hoidnud ära mükotoksiinide DON, HT-2 ja T-2 tekkimist terades (**III**).

- Väga tugevalt mõjutas ilmastik terade nakatumist mikroseentega (**II**), sealhulgas *Fusarium* spp. (**I, III**); ja mükotoksiinidega DON, HT2 ja T2 tekkimist terades (**III**).

- Katsetatud 15 *Fusarium* liigist vaid kaks liiki tootsid mükotoksiin fusareliine, *F. graminearum* ja *F. tricinctum* (**IV**). Uurimistöös selgus, et nimetatud *Fusarium* liikide mükotoksiin fusareliinide biosünteesi mõjutasid süsivesikute kogused, süsivesiku ja lämmastiku vorm, keskkonna happesus, temperatuur ja seene mütseeli vanus (**IV**).

Edaspidi uurimist vajavad teemad

Käesolevas töös tulid esile mitmed suunad, mis nõuavad pikaajalisemate uuringute tegemist. Uurimistöös selgus et taimekasvatustehnoloogia, kasvukoht ja ilmastik mõjutavad saagis *Fusarium* seente esinemist väga oluliselt. Siin oleks vajalik jätkata uurimistööga, selgitades erinevate kasvatustehnoloogiate võtteid millega ära hoida saakide rohke *Fusarium* seentega ja mükotoksiinidega saastumine. Kindlasti tuleb jätkata erinevate kasvatustehnoloogiliste mõjude selgitamist teraviljade saagis esinevatele mükotoksiinide DON, T-2 ja HT-2, ka teiste mükotoksiinide, näiteks zearalenoone, analüüsimisega. Samuti on vaja uurimisse tihedamalt siduda ilmastikuandmeid teraviljade kasvufaasides, et saaks prognoosida *Fusarium* seente ja mükotoksiinide saastumisriski.

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I

ORIGINAL PUBLICATIONS

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The effects of nitrogen rates and intercropping on the occurrence of *Fusarium* spp. on barley kernels

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Abstract. The aim of the field experiments was to compare the effect of nitrogen rates and intercropping on the occurrence of *Fusarium* spp. in barley kernels. The experiments were performed in Central Estonia (58˚33´N, 25˚34´E) in 2009 and 2010. The composition of fungi in spring barley kernels was found through isolation and subsequent sequence analyses of the internal transcribed spacer (ITS) region and morphological features. During the study, 13 species of micro-fungi were identified in the grain samples. The most common species of fungi found in barley were *Fusarium avenaceum, Fusarium poae*, as well as *Phoma pinodella*. The compositions of pathogenic fungi on Estonian barley kernels were affected by the level of nitrogen fertilization and growing on barley-pea intercropping. The study showed tendencies that barley-pea intercropping had fewer occurrences of *Fusarium* species than sole barley.

Key words: *Fusarium* spp., spring barley, nitrogen rates, intercropping

INTRODUCTION

The occurrence of *Fusarium* fungi in small grain crops causes poor quality of the grain and reduction of the yield. The fungal infection of the grain leads to shrunken kernels and contamination with toxic compounds like mycotoxins. The most common disease of cereals is the Fusarium head blight (FHB) caused mainly by five toxic pathogenic species, like *Fusarium graminearum*, *Fusarium culmorum*, *Fusarium poae*, *Fusarium sporotrichioides* and *Fusarium avenaceum* (Champeil et al., 2004; Terzi et al., 2014). The species have been thoroughly studied and investigated worldwide (Wagacha & Muthomi, 2007; Xu et al., 2007; Fernandez et al., 2008; Miedaner et al., 2008; Yli-Mattila, 2010; Fredlund et al., 2013). Moreover, the earlier studies in Estonia have found that the most common diseases on cereals are leaf blights and root rot, following snow

mould and FHB caused by *Microdochium nivale*, *Fusarium culmorum*, *Fusarium avenaceum*, *Fusarium sporotrichiella*, *Fusarium solani*, *Fusarium verticillioides* and *Fusarium oxysporum* (Lõiveke et al., 2004, 2008; Lõiveke, 2008).

The spread of pathogenic soil fungus *Fusarium* spp. is affected by various factors, like agro-meteorological conditions and agronomic practices. Weather conditions like warm or cool temperatures, low or high humidity and rare or intense precipitation during anthesis and maturity of cereals all strongly influence the occurrence of various *Fusarium* strains in different geographical locations (West et al., 2011; Bernhoft et al., 2012; Parikka et al., 2012; Popovski & Celar, 2013). Agronomic practices in the field have also an impact on the diversity and spread of *Fusarium* and other pathogenic fungi on grain. The results of previous studies have found that the preceding crops, fertilization, use of pesticides, crop variety, tillage and cultivation have an effect on the mycobiota in soil, plant and grain. In addition, there are reports that maize as preceding crop, cereals rich rotation, zero or minimal soil tillage and use of glyphosate favoured the spread of *Fusarium* fungi on cereals (Fernandez et al., 2008; Fernandez et al., 2009; Wegulo et al., 2015).

A good method of controlling plant diseases and *Fusarium* spp. is reasonable use of fertilizers in integrated plant production system. Moreover, nitrogen is the most important nutrient affecting disease development in cereal crops (Dordas, 2008). Hauggaard-Nielsen et al. (2008) summarized in the results of their study that intercropping of cereal with pulse improved the use of natural soil nitrogen and reduced the need for mineral nitrogen fertilizers. Cereal mixture with pulse also reduced incidence of the disease in the range of 20–40%. The effect of N fertilizers on occurrence of *Fusarium* spp. is not clear. Van der Burgt et al. (2011) and Krnjaja et al. (2015) reported that, enhancing the rate of N fertilization did not promote the infection of *Fusarium* in grain. On the other hand, Lemmens et al. (2004) and Suproniene et al. (2011) found that high rate of nitrogen increases occurrence of *Fusarium* fungi on grain. Still, little is known how intercropping of cereal with pulse affects the occurrence of *Fusarium* spp.

The aim of the current study was to compare *Fusarium* species composition in spring barley kernels under different rates of mineral nitrogen fertilizer and in pea-barley intercropping.

MATERIALS AND METHODS

The field experiment treatment

The field experiments were conducted in 2009 and 2010 on a *Podzoluvisol* (*PD*) (FAO, ISSS, ISRIC. 1998) in Central Estonia (58°33´N, 25°34´E). The experiment was laid out in randomized plots with design of four replicates. Plot size was 2.5 x 10 m. Winter wheat was the previous crop. In the autumn the experimental plots were ploughed. Barley variety 'Anni' and pea variety 'Clarissa' were grown in the experiments. Mineral complex fertilizer (Skalsa N5–$P_2O_5$10–K_2O25, dose of fertilizer 250 kg ha^{-1} and nitrogen amount 12.5 kg ha^{-1}) was sown together with the seeds for all the treatment plots. The seeds were sown in first decade of May. The ammonium nitrate fertilizer was added as top dressing in the beginning of tillering (GS 21).

The rates of nitrogen (N) were as follows:

1) barley with added ammonium nitrate (dose of fertilizer 312.5 kg ha^{-1}) – N_{120} kg ha^{-1};

2) barley with added ammonium nitrate (dose of fertilizer 138 kg ha^{-1}) – N_{60} kg ha^{-1};
3) barley without added ammonium nitrate – N_{40};
4) barley-pea intercrops without ammonium nitrate – BP.

We presumed that the soil of barley-pea intercrop contains naturally around 40–50 kg of free N per ha (Freyer, 2003). The sowing rate of the sole barley was 550 seeds per m^2 and in the intercrop 120 barley and 80 pea seeds per m^2. Herbicide Butoxone (active ingredient MCPB 400 g l^{-1}) was used for weed control in dosage of 3.8 l ha^{-1}, and no fungicides were used for disease control. After harvesting in the end of August the seeds were dried to 14% of standard moisture, sorted and a 1.5 kg sample was taken from each treatment. Samples were stored at –4 °C until laboratory analysis.

The isolation and identification of fungi

For isolation and identification of fungi the surface of barley grains were sterilized for 5 minutes with 1% of sodium hypochlorite (NaOCl) and rinsed twice with sterile distilled water. According to Abildgren et al. (1987), the 100 kernels were placed in Petri dishes containing CZID medium (35 g of Czapek Dox broth, 15 g of Agar, 1 ml of trace solution, 1 ml of dichloran, 1 ml of Chloramphenicol in 1 L of MQ water, autoclaved at 121 °C for 15 min). The plates were incubated at 20 °C in the dark (8 h) and in the light (16 h) cycle. After seven days of incubation, the fungi were transferred to Potato Dextrose Agar (PDA: 39 g of Potato Dextrose Agar, 1 mL of trace solution, 1 L of MQ water, autoclaved at 121 °C for 15 min) and the inoculated Petri dishes were kept in the dark for 14 days at 28 °C. Fungi determined morphologically accordingly to Samson et al. (2002) and Leslie and Summerell (2006). After incubation the DNA of all fungi was purified with the Ultra Clean Microbiol DNA Isolation Kit (MOBio Inc.CA) according to the manufacturer´s protocol. The internal transcribed spacer (ITS) region of the rDNA gene (S) was amplified using fungal universal primers: ITS1 (5´TCCGTAGGTGAACCTGCGG) and ITS4 (5´TCCTCCGCTTATTGATATGC) (White et al. 1990). The thermal cycler profile was used with denaturation at 98 °C for 5 min; followed by 30 cycles of amplification with denaturation at 98 °C for 1 min, primer annealing at 52 °C for 1 min, extension at 72 °C for 5 min, followed by cooling at 4 °C. Next, the 35 µl of PCR product was taken and cleaned further with Qiaquik PCR Purification Kit (Qiagen). The Sample Submission guide of EUROFINS MWG Operon was followed to prepare DNA to sequence the PCR products. The obtained DNA sequences were analysed using the UNITE database (https://unite.ut.ee) (Koljalg et al., 2013). The BLAST searching system at GenBank sequence database http://blast.ncbi.nlm.nih.gov/ was used to identify the fungi strains.

Weather conditions

The weather conditions during vegetation period (from May to August) in 2009 and 2010 were different (Table 1). In 2009, the average temperature for four-month period was 2.4 °C degrees cooler than in 2010. The overall amount of precipitation over the years did not differ significantly, being 332.9 mm in 2009 and 316.2 mm in 2010. However, the amount of precipitation during the vegetation period distributed unevenly. In 2009, June was rainy (sum of precipitation 85.3 mm) and July extremely rainy (sum of precipitation 135.6 mm). In 2010, July was dry (sum of precipitation 42.8), but August was very rainy (sum of precipitation 143.2 mm).

Table 1. Weather conditions in 2009 and 2010 in Central Estonia

		Day of rainfall		Sum of precipitation, mm		Average air temperature, °C	
Month	Decade	2009	2010	2009	2010	2009	2010
May	I	3	7	5.2	38.2	10.5	7.3
	II	2	4	6.0	4.8	9.9	17.2
	III	3	5	5.6	9.8	13.9	12.5
		8	**16**	**16.8**	**52.8**	**11.4**	**12.3**
June	I	4	3	48.7	18.6	11.3	13.9
	II	9	6	35.6	27.4	13.1	13.8
	III	2	2	1.0	31.4	17.3	16.3
		15	**11**	**85.3**	**77.4**	**13.9**	**14.7**
July	I	5	3	46.2	16.0	16.1	20.6
	II	6	3	47.6	9.2	17.9	23.6
	III	8	4	41.8	17.6	17.4	22.8
		19	**10**	**135.6**	**42.8**	**17.1**	**22.3**
August	I	5	3	20.6	10.6	17.0	21.4
	II	7	6	56.6	68.4	15.2	19.8
	III	4	7	18.0	64.2	15.0	14.6
		16	**16**	**95.2**	**143.2**	**15.7**	**18.6**
Total/average		**58**	**53**	**332.9**	**316.2**	**14.6**	**17.0**

The statistical analysis

The frequency of occurrence isolated fungal species calculated as number of isolated species divided total number of isolated species. The percentage occurrence of fungal species was found as the number of kernels in what species occurred divided on total number of kernels multiplied by 100. Analysis of variance (ANOVA, SAS 2002) used for determining effects of nitrogen rates and intercropping on the occurrence of *Fusarium* spp. on the barley kernels. The significance threshold was set at $p < 0.05$.

RESULTS AND DISCUSSION

During the study in total 85 isolates and 12 species of fungi were identified in the spring barley kernels, while the occurrence of *Fusarium* fungi was appreciable different in both years of the study (Table 2). The seven fungal genera were identified in 2009 and two fungal genera in 2010. In 2009, *Fusarium* spp. were isolated from 38% of kernels, whereas the occurrence of other pathogenic fungi was 62%. While in 2010, *Fusarium* was the common genus (96%) isolated from barley kernels. Our results confirm the findings of several researchers: that the weather factors have a significant effect on the occurrence of *Fusarium* spp. and other pathogenic fungi in kernels (Blandino et al., 2008; Parikka et al., 2012; West et al., 2012). Research of Blandino et al. (2008) showed that the climatic factors influenced the presence of fungal species; moreover, the rate of nitrogen application had an effect on fungal infection in maize kernels. Parikka et al. (2012) suggested that warm weather with more rainfall near harvest increases the spread of *Fusarium* spp., while high temperature, dryness and intense rainfall cause plant stress and favour of *Fusarium* infection on cereals in Northern Europe. West et al. (2012) concluded that the weather conditions together with the effect of agronomic practices influenced the interaction of pathogenic fungal species. Consequently, our study confirmed that warmer vegetation period and rainy harvesting season as it was in 2010

favoured the contamination of kernels with *Fusarium* fungi. On the contrary, in 2009, the moderate air temperatures with high rainfalls in July increased the occurrence of other pathogenic fungi that may suppress *Fusarium* fungi.

Table 2. Frequency of occurrence (%) of isolated fungal species from spring barley kernels in 2009 and 2010

Fungal species	Frequency (%)		
	2009	2010	Mean
Arthrinium sacchari	2.8	2.0	2.4
Alternaria infectoria	2.8	0.0	1.2
Epicoccum nigrum	8.3	0.0	3.5
Fusarium avenaceum	11.1	51.0	34.1
Fusarium equiseti	16.7	2.0	8.2
Fusarium oxysporum	0.0	6.1	3.5
Fusarium poae	2.8	16.3	10.6
Fusarium sporotrichioides	0.0	10.2	5.9
Fusarium tricinctum	5.6	10.2	8.2
Microdochium bolleyi	13.9	2.0	7.1
Phoma pinodella	22.2	0.0	9.4
Parastagonospora nodorum	13.9	0.0	5.9

Unlike the weather condition, the impact of nitrogen on the occurrence of *Fusarium* and other pathogenic fungi was less obvious. However, we still found that the effects of N rates and intercropping were statistically significant. In this study, the occurrence of micro-fungi on the barley kernels was higher at N_{120} and N_{40} compared to N_{60} rate and intercropping treatment (Fig. 1). The frequency of occurrence of micro-fungi on the barley kernels in the intercropping (BP) treatment was 1.8 times lower than in N_{40} and 1.6 times lower than in N_{120} treatment. Several researchers concluded that high rates of nitrogen fertilizer application increased *Fusarium* infection level in grain (Suproniene et al., 2012; Lemmens et al., 2004, van der Burgt et al., 2011). Lemmens et al. (2004) explained that increasing N input changes the plant canopy density, which in turn influences microclimatic conditions in plant-soil environment and delays the flowering period, therefore creating favourable conditions for infection. Probably the low or high rate of nitrogen leads the plants to stress that makes them more susceptible to *Fusarium* spp. and other pathogenic micro-fungi (Blandino et al., 2008). The highest occurrence of micro-fungi in the rate of N40 was probably due to the stress caused by nutrient deficiency; also, the plant height (unpublished data) was lower than in other treatments. It may be caused by the fact that the macro-conidia from Fusarium spp. can splash-disperse as high as 40–60 cm vertically from the source (Jenkinson & Parry, 1994; Lemmens et al., 2004), thereby contaminating the barley kernels. Review article by Dordas (2008) discusses the effect of N on contamination of pathogen depending on the type of fungi. It says that although high N application increases the severity of infestation by obligate parasitic fungi (such as Puccinia spp. and other diseases), it also decreased the infestation by facultative fungi (such as Alternaria spp., Fusarium spp.) In addition to interactions, repression and competitions are held between the different microorganisms, where the host plant responds with a complex of biochemical reactions, thereby becoming more susceptible or resistant to the pathogens (Dordas, 2008). The characteristic of plant density can also influence a distribution of *Fusarium* and other

fungi whereas in tight plant spacing humid conditions could remain for longer than in a sparser intercrop. In this study, we found tendencies that the high occurrence of *Fusarium* fungi corresponds with low rate of the N_{40}, followed the top dressing treatment rates of the N_{120} and N_{60}.

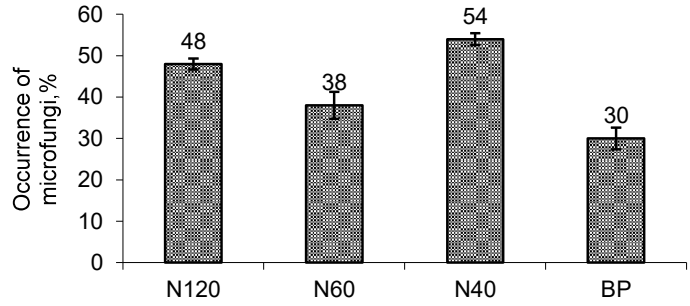

Figure 1. The average frequency of occurrence of micro-fungi in barley cropped under different nitrogen levels and barley-pea (BP) intercropping in 2009 and 2010.

Our study revealed that *Fusarium avenaceum* and *Fusarium poae* were the most common fungal species isolated from barley kernels (Table 3). *Phoma pinodella* and *Microdochium bolleyi* had high frequency of other isolated fungi. *Fusarium lateritium* dominated in kernels from all the different rates of nitrogen and also in intercropping. *Fusarium poae* was detected in the kernels from the mineral nitrogen rate and the rate of N_{40} but not in intercropping barley kernels. *Fusarium sporotrichioides* identified only in kernels from the nitrogen rate of N_{120} and N_{40}, but not in N_{60} and intercropping kernels. In barley kernels from intercropping treatment we identified only *Fusarium lateritium* compared at the rates of N_{120}, N_{60} and N_{40}. Other identified micro-fungi also showed interesting tendencies. The pathogenic fungi *Parastagonospora nodorum* occurred mainly in the rate of N_{120} in barley kernels. It was statistically significant that pathogenic fungus *Phoma pinodella* dominated only in kernels from intercrop. However, our results showed that the intercropping of cereal with pea might be an option to decrease the infestation of barley grain with *Fusarium* fungi. In addition, the occurrence of micro-fungi on grain decreased in moderate mineral nitrogen application as N_{60} compare with high rate and very low rate of nitrogen.

In this study the species *Fusarium avenaceum* dominated in the kernels from all treatments. The *Fusarium* species like *Fusarium poae* and *Fusarium sporotrichioides* were modestly represented in top dressing mineral nitrogen type and N_{40} but not in intercropping.

Results from this study showed tendencies that in the barley kernels from intercropping treatment, *Fusarium* species occurred less than in mineral nitrogen top dressing fertilization treatment. Likewise van der Burght et al. (2011) found significant positive effect of top dressing on the FHB infected seeds. We consider that compared to the intercropping of barley with pea the top dressing at sole barley favourably affected the grain contamination by *Fusarium* and in low fertilization rate as N_{40} caused stress in

plants, thereby making them more susceptible to the *Fusarium* and other pathogenic fungi. Hauggaard-Nielsen et al. (2001) explained that in intercropping the barley is capable to take more soil mineral nitrogen in contrast to sole barley. This explains why barley mixed with pea does not starve from lack of nitrogen like barley in the N_{40} treatment. Thus, lower occurrence of *Fusarium* spp. in intercropped kernels compared with other types of treatments may be due to moderate supply of nitrogen as well as sparse plant density in this treatment.

Table 3. The probability of occurrence of fungi species in barley cropped under different rates of nitrogen and barley-pea (BP) intercropping in 2009 and 2010

Species	N_{120}	N_{60}	N_{40}	BP
Arthrinium sacchari	ns	no	ns	no
Alternaria infectoria	ns	ns	ns	no
Epicoccum nigrum	ns	no	ns	no
Fusarium avenaceum	0.004	0.001	0.002	0.006
Fusarium equiseti	ns	ns	ns	ns
Fusarium oxysporum	ns	no	ns	no
Fusarium poae	0.004	0.05	0.04	no
Fusarium sporotrichioides	0.05	no	0.004	no
Fusarium tricinctum	ns	no	ns	ns
Microdochium bolleyi	ns	ns	ns	no
Phoma pinodella	no	no	no	0.002
Parastagonospora nodorum	0.001	no	ns	no

no – not occurred

ns – not statistically significant at the $p < 0.05$

CONCLUSIONS

Based on the results of study we found tendencies that the higher air temperature and rainy weather during the maturity of barley favourably influenced the infestation ofkernels by *Fusarium* spp. The moderate temperatures and more than average precipitation in 2009 were favourable conditions for *Phoma pinodella*, *Microdochium bolleyi* and other mould. Frequency of occurrence of *Fusarium* was 36% in 2009. In warmer and rainy 2010 *Fusarium* species were most abundant (frequency of occurrence of *Fusarium* 96%) fungi in the barley.

The barley-pea intercropping and investigated rates of mineral nitrogen in barley influenced the occurrence of *Fusarium* and other micro-fungi differently.

The *Fusarium avenaceum* was dominant in all treatments. Experiments revealed tendencies that *Fusarium poae* and *Fusarium sporotrichioides* were mainly represented in the mineral fertilizer top dressing treatments. The frequency of occurrence of micro-fungi in the intercropping treatment was lower as mineral nitrogen fertilizer treatments.

Fusarium spp previously not been investigated in kernels from intercropping and nd under two years experiments not enough to make definitive conclusions. We found interesting tendencies, but more experiments need for final conclusions.

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II

Akk, E., Lõiveke, H., Edesi, L., Kütt, M.-L., M., Lauringson, E., Kastanje, V. 2013.

Formation of the abundance of microfungi on the barley grain grown as pure and mixed crops in Central and North Estonia.

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Formation of the abundance of microfungi on the barley grain grown as pure and mixed crops in Central and North Estonia

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Abstract. For centuries barley has been an important food crop for mankind. It is important to produce crops that are of good quality and safe to human and animal organisms. However, pathogenic fungi in cereals cause health problems both to humans and animals. In Estonia the microbiological quality of cereals has been studied to identify *Fusarium* species in feed cereals. Still, the relationship between the agro-ecological conditions and the total abundance of moulds and yeasts on grain has been studied little in Estonia. In 2009 and 2010, we carried out field trials in the experimental station of the Estonian Research Institute of Agriculture in North Estonia (59°18′N, 24°39′E) and in the experimental station of Olustvere School of Service and Rural Economics in Central Estonia (58°33′N, 25°34′E). The variants of the experiment were barley in pure crops with added ammonium nitrate 120 kgN ha^{-1}, barley in pure crops with added ammonium nitrate 60 kgN ha^{-1}, barley in pure crops with no added ammonium nitrate, and barley–pea mixed crop. After harvesting, the grain was dried to 14% of moisture and grain samples were taken from each trial variant. The abundance of moulds, yeasts, and *Fusarium* spp. was determined in grain samples using the dilution method. The impact of the levels of nitrogen, location of the trial site, and year (weather conditions) on the abundance of moulds, yeasts, and *Fusarium* spp. was studied. The common genera of moulds identified were *Cladosporium*, *Acremonium*, and *Fusarium*. According to our results, yeasts were the most common fungi on barley grains. In North Estonia the abundance of moulds was lower compared to Central Estonia. The abundance of fungi was not affected by different levels of nitrogen or whether barley had been grown as a pure crop or a barley–pea mix. The weather conditions had the greatest impact on the abundance of microfungi.

Key words: cereal, moulds, yeasts, *Fusarium* spp., quantitative method.

INTRODUCTION

For centuries barley has been an important food crop for mankind. Today most of the barley grown is used in the beer and malt industry, but also as animal and human food. In general, about two-thirds of the world's barley production has been used for feed, one-third for malting, and 2% for the food industry (Newman & Newman, 2006).

Environmental variations, temperature, and humidity have an important impact on the yield and quality of cereals (Šarić et al., 1997; Lõiveke et al., 2003; Ingver et al., 2010). Furthermore, the agronomic practices may influence the yield and microbiological quality of the crop. The effect of tillage practices, fertilizer treatments, cropping system, and crop protection on *Fusarium* spp. and on levels of mycotoxins has been studied mainly on wheat (Champeil et al., 2004; Fernandez et al., 2008; Lori et al., 2009; Vujanovic et al., 2012). Previous studies on barley have focused on the resistance of varieties to ear blight (Legzdina & Buerstmayr, 2004), concentration of mycotoxins (Mankevičienė et al., 2006; Edwards, 2007; Ibáñez-Vea et al., 2012), and the effect of *Fusarium* spp. infection on the malting process (Wolf-Hall, 2007).

In Estonia precipitation exceeds evaporation. Strongest rainfalls occur from July to November (Estonian Second National Report…, 1998). Therefore, in most years the periods of anthesis, grain filling, and harvesting of cereals coincide with the rainy period. In Estonia spring barley cultivation is prevalent because winter barley is not cold resistant in our climate (Bothmer et al., 2003).

Nowadays increasingly more attention is paid to food and feed safety and the quality of crops. However, microorganisms, such as moulds, may cause the spoilage of grain, which then becomes hazardous for humans and animals (Richard, 2007). A high amount of moulds in malt can cause toxicity of raw material in the brewing industry (Medina et al., 2006). Therefore, investigation of pathogenic moulds deserves high attention, and early discovery of infestation is important (Placinta et al., 1999; Siegel & Babuscio, 2011). Several researchers have studied the microbiological quality of barley focusing on moulds such as *Alternaria* spp., *Aspergillus* spp., *Penicillium* spp., and *Fusarium* spp. and their toxins (Rabie et al., 1997; Yli-Mattila, 2011).

In Estonia the microbiological quality of cereals has also been studied to identify *Fusarium* species in feed crops. Lõiveke et al. (2003) reported that *F. avenaceum*, *F. ventricosum* (synonym of *F. solani*), *F. sporotrichioides*, and *F. poae* are the most common *Fusarium* species on feed cereals. They rated 14–46% of the barley crop samples infected. However, the relationship between the agro-ecological conditions and the total abundance of moulds and yeasts on barley grain has been little studied in Estonia.

The general method applied for analysing grain quality is the assessment of the total abundance of moulds and yeasts on cereals. The results of the quantitative assessment show whether the food or feed is contaminated by microfungi (Schmidt-Lorenz, 1980; Baumgart & Firnhaber, 1993).

Although the capacity of well-equipped laboratories to identify and quantify mould species on grain is high, the general abundance of moulds and yeasts on crop fields has been little studied. The aim of this study was to identify, using a quantitative method, the impact of the level of nitrogen, field location, and year (weather conditions) on the abundance of moulds, yeasts, and *Fusarium* spp. on barley grain.

MATERIALS AND METHODS

The field experiments with barley (*Hordeum vulgare* L.) were carried out in 2009–2010. In North Estonia the field trials were performed in the Saku experimental station of the Estonian Research Institute of Agriculture (59°18′N, 24°39′E), where Cambisols (CM) are the prevailing soil type (FAO, ISSS, ISRIC, 1998). In Central Estonia the field experiments were made in the experimental station of Olustvere School of Service and Rural Economics (58°33′N, 25°34′E); there the prevailing soil type is Podzoluvisols (PD) (FAO, ISSS, ISRIC, 1998). In both locations the field trials were sown in the first 10 days of May and harvested in the last 10 days of August. Spring barley variety Anni and pea (*Pisum sativum* L.) variety Clarissa were used in both experiments.

During the sowing of barley pure crops and barley–pea mixed crops the complex fertilizer Skalsa with a low nitrogen content was added at a rate 250 kg ha^{-1} (nutrients N10–$P_2O_5$50–K_2O63 kg ha^{-1}). At the stem stage of barley, the pure crop received an additional ammonium nitrate fertilizer. The variants of the experiment with ammonium nitrate were (1) N120: barley pure crops with added ammonium nitrate 120 kgN ha^{-1} (quantity of fertilizer 320 kg ha^{-1}); (2) N60: barley pure crops with added ammonium nitrate 60 kgN ha^{-1} (quantity of fertilizer 145 kg ha^{-1}); (3) N0: barley pure crops without ammonium nitrate; and (4) BP: barley–pea mixed crop without ammonium nitrate. However, it was taken into account that the soil of barley–pea mixed crop contains about 40–50 kg of free N per ha (Freyer, 2003). For weed control the crops (stem stage of barley and 3–9 leaves stage of pea) were treated with the herbicide Butoxone (1520 g ha^{-1} MCPB in active ingredient), dosage 3.8 L ha^{-1}.

The field trials were harvested with a combine harvester. The grain was dried to 14% of standard moisture, sorted, and a 1.5-kg average sample was taken per every field experiment variant. Microbiological analyses were carried out in the Laboratory of Plant Health and Microbiology at the Agricultural Research Centre, Estonia. The preparation of samples was performed according to the standard EVS-EN ISO 6887-1:2001 (Estonian Centre for Standardisation, 2001). The dilution method was used to determine the abundance of moulds, yeasts, and *Fusarium* spp. in each sample. The moulds and yeasts were grown on Czapek agar (ICC Standard No. 146, 1992) and *Fusarium* spp. fungi on Nash and Snyder selective medium (Booth, 1971; Gerlach & Nirenberg, 1982). The abundances of moulds, yeasts, and *Fusarium* spp. were presented as the number of colony-forming units per 1 g of dry grain (CFU g^{-1} of dry grain).

The microbiological quality of barley grain was evaluated using the indicators for food and feed established by Schmidt-Lorenz (1980) and Baumgart & Firnhaber (1993). According to these indicators, on grain of normal quality the abundance of moulds or yeasts must not exceeded 3×10^4 CFU g^{-1} of dry grain in food grain and 4×10^4–8×10^4 CFU g^{-1} of dry grain in feed grain (Lõiveke et al., 2004).

The meteorological data were obtained from the observation points of the Estonian Meteorological and Hydrological Institute.

The statistical analyses were performed using Microsoft Excel 2003. ANOVA test was used to compare the differences between variants at the 95% probability.

RESULTS AND DISCUSSION

Weather conditions

During the two vegetation periods the air temperatures differed significantly but both trial years were rich in precipitation. During the growing season of 2009 the air temperatures in North and Central Estonia were similar and favourable for plant growth. In 2010 the growing season was warm. The mean air temperature was 3.3 °C above the region's long-term average (14.4 °C) in Central Estonia and exceeded the region's long-term average (13.8 °C) by 2.1 °C in North Estonia.

The major difference between the two regions during the study period was the registered precipitation amounts. In Central Estonia the amounts of precipitation during the vegetation seasons of 2009 and 2010 were 120% and 114%, respectively, of normal long-term average (277 mm). The precipitation amounts for North Estonia were 74% and 67%, respectively, of normal long-term average (268 mm). Besides, the temporal distribution of precipitation was different in North and Central Estonia.

In July 2009 North Estonia suffered from drought and normal rainfall came only in August. In Central Estonia it rained a lot in both months. The July of 2010 was very rainy in North Estonia while in Central Estonia rainfalls were scanty. In August 2010 the situation was the opposite: it was dry in North Estonia while in Central Estonia the amount of precipitation reached almost the double monthly norm. During the two experimental years the rainfall in July and in August made up 88% of the monthly norm in North Estonia and 129% in Central Estonia.

Microbiological composition

Our results of microbiological analysis of grain showed that the microbiological composition differed greatly by the groups of microfungi (Fig. 1). Olstorpe et al. (2010) also detected that the microbial flora in barley grain varies considerably and the microbial population from the field changes during storage. In our study the common genera of moulds were *Cladosporium*, *Acremonium*, and *Fusarium*. *Cladiosporium* spp. are among the most common saprophytes on cereals. *Fusarium* spp.

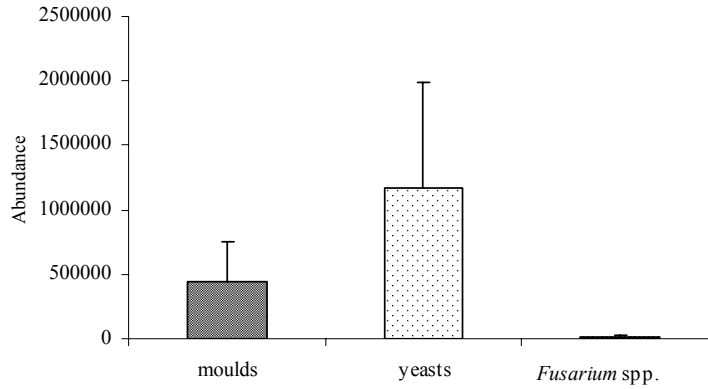

Fig. 1. Average total abundance of moulds, yeasts, and *Fusarium* spp. (CFU g^{-1} of dry matter) on barley grain in 2009 and 2010.

are pathogens, which produce mycotoxins on cereal grains (Suproniene et al., 2011). *Acremonium* spp. appear in soil and associate with plants (Domsch et al., 2007; Vujanovic et al., 2012). In 2009 moulds were the most common microfungi group on barley grain while in 2010 yeasts (species not determined) dominated (Fig. 1). The overall abundance of yeasts on barley grain was higher compared to the overall abundance of moulds and *Fusarium* spp. Yeasts increase the pH and harm the storage stability of cereals (Middelhoven & van Baalen, 1988). However, yeasts may also have positive effects, inhibiting the growth of moulds on the moist cereal grains (Passoth & Schnürer, 2003; Druvefors & Schnürer, 2005) and enhancing the nutritional value of animal feed (Bui & Galzy, 1990). Yeasts are useful and necessary for the fermentation processes of cereals, especially in cereal silage and in the beer industry (Fleet, 2007; Olstorpe et al., 2010).

Compared with the abundance of moulds and yeasts that of *Fusarium* spp. was relatively low, constituting only 1.6% of the total abundace of moulds and yeasts. Krysinska-Traczyk et al. (2007) found that *Fusarium* strains have been very rarely compared to the common species of moulds and yeasts. The reason may be that some yeasts and *Acremonium* spp. inhibit the growth and progression of *Fusarium* spp. on the grain (Vujanovic et al., 2012). However, in 2010 the abundance of moulds, yeasts, and *Fusarium* spp. was higher compared to the 2009 trial (Fig. 2a). An increase of precipitation in July during the time of anthesis and seed formation may significantly raise the abundance of moulds, yeasts, and *Fusarium* spp. on the grains. Furthermore, a high precipitation amount during the growing season, particularly during the pre-harvest period, could increase the abundance of moulds on the grain (Sutton, 1982). This means that weather conditions are extremely important for the development of various microfungi (Torp & Nirenberg, 2004; Uhlig et al., 2007; Tirado et al., 2010; West et al., 2012). The development of yeasts was favoured by 2–3 degrees higher than average air temperatures in 2010. It was also observed that an increase of yeasts could cause

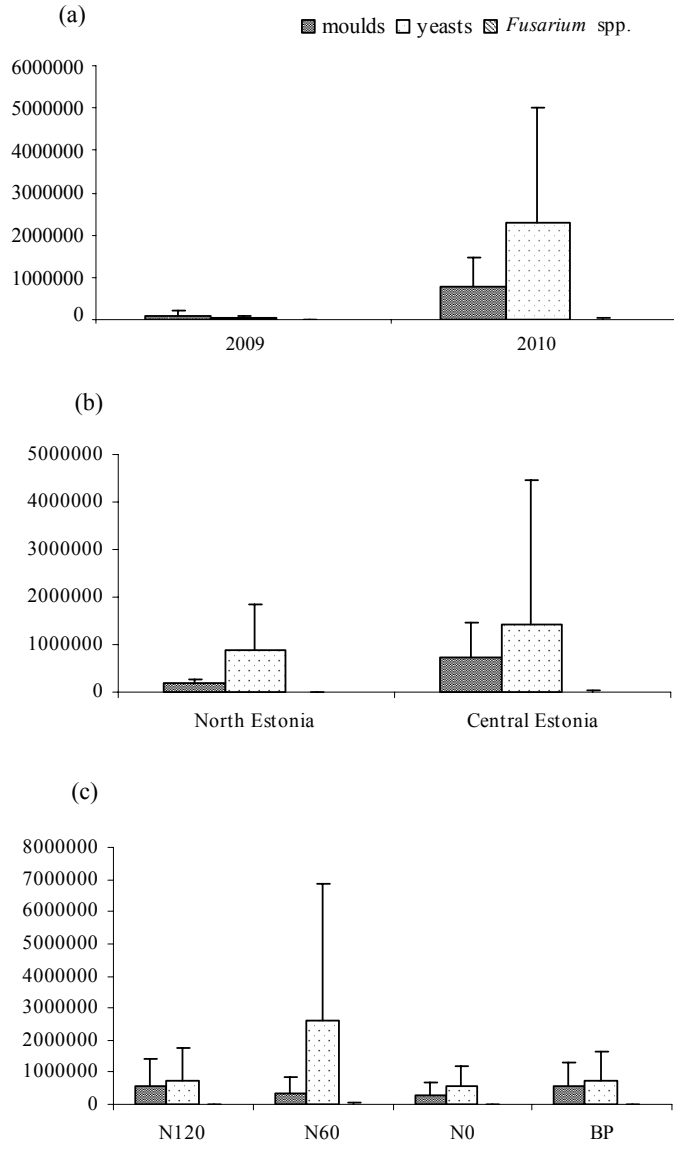

Fig. 2. Comparison of the abundance (CFU g^{-1} of dry grain) of moulds, yeasts, and *Fusarium* spp. between (a) 2009 and 2010, (b) trial locations, and (c) different amounts of nitrogen applied at the stem stage of barley (N120 = 120 kgN ha^{-1}, N60 = 60 kgN ha^{-1}, N0 = no ammonium nitrate added, BP = barley–pea mixed crop).

a decrease of moulds. Despite the erratic precipitation in both habitats during 2010, a high abundance of yeasts was registered in both test locations.

The results a two years of trial showed that the average abundance of moulds in barley samples from Central Estonia was four times higher and the abundance of yeasts two times higher compared to the samples from North Estonia (Fig. 2b). As we used the same sowing time, fertilization, varieties, crop protection, and harvesting time in different locations, this indicates that the weather conditions in the growth location were more important for the formation of the abundance of moulds and yeasts than agronomic practices. In addition, the formation of the microbial composition on grain is significantly affected by interactions between moulds and yeasts (Fernandez et al., 2008; Vujanovic et al., 2012).

The differences between the abundances of microfungal groups from trials with different nitrogen levels were not statistically significant (Fig. 2c). The highest abundance of yeasts was observed in the N60 variants whereas the highest average abundance of moulds was shown by the BP and N120 variants. In the trial variant N60 the highest percentage of yeasts (87%) and the lowest percentage of moulds (12%) were observed. In the trial variants where the percentage of yeasts in the microbiological composition was lower (55–65%), the percentage of moulds was higher (about 33–44% of the total fungal abundance). This indicates a potential positive effect of yeasts in reducing moulds in the microbiological composition of barley grain. Consequently, the effect of soil nitrogen content on the abundance of microfungal groups was less significant than the impact of the weather. Apparently, the use of different dosages of nitrogen in the cultivation of barley as a pure crop or a mixed crop with pea did not have a direct impact on the microbiology of barley grain in Central and North Estonia. The effect of soil nitrogen content and abundance of moulds, particularly *Fusarium* spp., and plant diseases has been studied by several researchers. In some cases the reports show that a low level of nitrogen tends to promote the infection by *Fusarium* spp. while a high level of nitrogen reduces the grain contamination with *Fusarium* spp. (Yang et al., 2010). However, in another study exactly the opposite conclusion was drawn: the high levels of nitrogen were found to favour the contamination of grain with *Fusarium* spp. (Champeil et al., 2004).

Another important aspect is agro-ecological conditions, which may also affect the content of moulds, especially *Fusarium* spp., on cereals. Through the implementation of crop rotation, moderate fertilization, and use of pesticides the development of the *Fusarium* fungi as well as the emergence of mycotoxins could be reduced (Edwards, 2004). In our study the abundance of *Fusarium* spp. was not affected by the level of nitrogen. We found that the effect of weather conditions during the growing season in the growth location was more important for the abundance of microfungi than the effect of fertilization with nitrogen: we did not observe any significant impact of soil nitrogen content on the abundance of moulds, yeasts, and *Fusarium* spp. on barley grain.

In Estonia no guidelines have been established for counting moulds and yeasts on grain. To assess whether the microbiological quality of barley grain is suitable for food or feed the indicators of Schmidt-Lorenz (1980) and Baumgart & Firnhaber

Table 1. Suitability of barley from the experiment for microbiological quality of food and feed according to Schmidt-Lorenz (1980) and Baumgart & Firnhaber (1993) indicators for cereal crops (CFU g^{-1} of dry grain)

Location	2009		2010	
	Moulds	Yeasts	Moulds	Yeasts
Central Estonia	5.9×10^3	6.56×10^4	13.7×10^5	28.2×10^5
North Estonia	14.9×10^4	3.5×10^4	19.4×10^4	17.5×10^5

Indicator for food 3×10^4; for feed 4×10^4–8×10^4.

(1993) are used. To meet the quality standards of food grain and feed grain the abundance of moulds or yeasts must not exceed 3×10^4 CFU g^{-1} of dry matter and 4×10^4–8×10^4 CFU g^{-1} of dry matter, respectively (Lõiveke et al., 2004). According to microbiological quality indicators, in 2010 the barley grain did not correspond to the Schmidt-Lorenz (1980) and Baumgart & Firnhaber (1993) indicators for feed and food. In 2009 the barley grain grown in Central Estonia met the requirements for feed but not for food (Table 1).

The saprophytic and pathogenic fungi on barley kernels induce various symptoms. Although seed damage begins before harvest, it may increase if grains are stored under moist or wet conditions or they are harvested late (Wiese, 1987). At harvest and storage of cereals, spore containing material and humidity combined with temperature are factors affecting mould and yeasts formation in cereals (Schrödter, 2004).

CONCLUSIONS

The trials of this study were conducted under contrasting weather conditions; therefore we cannot draw any definitive conclusions on the basis of the two-year results. Still, our results showed that the most frequent fungi on the analysed barley grain were yeasts. The abundance of moulds on barley was lower in North Estonia than in Central Estonia. The total abundance of microfungi was most affected by the weather conditions during the vegetation season in both locations. Growing barley at different soil nitrogen levels in pure crops and mixed crops with pea did not have any impact on the amount of microfungal groups in the barley yield.

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III

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Application of widely used fungicides does not necessarily affect grain yield, and incidence of Fusarium spp. and mycotoxins DON, HT-2 and T-2 in spring barley in northern climates

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**in memoriam

Abstract

Fungicides are widely used to reduce *Fusarium* infections and grain contamination by mycotoxins and increase the yield in cereals, but the efficacy of fungicide treatments in varying climates has not been systematically explored. Field experiments with Estonian spring barley (*Hordeum vulgare* L.) cv. 'Maali' were carried out in three successive years 2012–2014 with strongly varying weather conditions to study the effects of three fungicides, Folicur (active ingredient tebuconazole), Falcon Forte (prothioconazole, tebuconazole, spiroxamine) and Archer Top (fenpropidin, propiconazole), on the yield, incidence of *Fusarium* spp. and on the contamination of grain with mycotoxins DON, HT-2 and T-2. The fungicides were sprayed once a year at spring barley flowering time. The weather conditions during the three years of study were extremely different. The content of mycotoxin DON, HT2 and T2 was low. The spraying with fungicides had not a clear effect on the barley yield and 1 000 kernel weight, and the study year was primarily the main factor that affected barley yield ($p<0.05$) and 1 000 kernel weight ($p<0.05$). The impact of year together with fungicide treatment had a significant effect on the incidence of *Fusarium* spp. ($p<0.05$) and on the incidence of mycotoxin DON in barley kernels ($p<0.001$), but did not have a clear effect on the incidence of mycotoxins HT2 and T2.

Key words: spring cereal, pesticide, moulds, trichothecenes

1 Introduction

Spring barley is the most widely grown spring cereal crop in Estonia. In 2016–2018 the spring barley growing area was 36% of all cereal crops growing area and 56% of the spring cereals area (Statistics Estonia, 2019). Spring barley for Estonia is an economically important crop since, in 2006, 63% of barley grain was exported. In 2016 in Estonia, 36% spring barley was used as animal feed, 5% as seed and 0.3% of barley was used for human consumption (Statistics Estonia, 2016). On average, 2% of globally produced barley is used directly as human food, 25% is used for malting and brewery industry and the main part of the barley is used for animal feed (Baik and Ullrich, 2008). In spring

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barley, the *Fusarium* Link ex Fr. causes worldwide disease and grain contamination with mycotoxins (Parikka et al., 2012; Nielsen et al., 2014; Horky et al., 2018). In Northern Europe, the *Fusarium* head blight (FHB) caused mainly by *F. graminearum sensu stricto*, *F. culmorum*, *F. poae*, *F. sporotrichioides*, *F. langsethiae*, *F. tricinctum* and *F. avenaceum*, reduces grain quality and the usefulness of grain for food and feed purposes by producing a variety of mycotoxins, of which most common are deoxynivalenol (DON), T-2, HT-2 and nivalenol (NIV) (Yli-Mattila et al., 2011, 2013; Hietaniemi et al., 2016). In earlier studies in Estonia it was found that *Fusarium* spp. were present in 79% of feed barley samples and on average 29% of spring barley grains were contaminated with *Fusarium* spp. Dominant species identified in spring barley grains were *F. avenaceum*, *F. sporotrichioides*, *F. poae*, *F. oxysporum*, *F. solani* and *F. culmorum* (Lõiveke et al., 2003). In 2006 and 2007, mycotoxins were present in 41% and 66% of feed cereal samples respectively, and it was demonstrated that the mould count and the occurrence of *Fusarium* spp. increases with increasing total precipitation and precipitation frequency during the flowering and pre-harvest time of the cereals (Lõiveke et al., 2008). Field trials with wheat showed that the use of the fungicides in moist and wet vegetation period decreased the count of moulds and *Fusarium* spp. in grain (Lõiveke, 2004). The results of the study confirmed that Estonian climatic conditions are favourable for mycotoxin production in cereals during vegetation period, but no correlation was found between the mould count, *Fusarium* spp. count and accumulation of mycotoxins (Lõiveke, 2004). The use of chemical control measures such as fungicide spray at cereal anthesis stage has been well investigated and is recommended for prevention of mycotoxin accumulation in grain (Wegulo et al., 2015). Lõiveke et al. (2004) investigated the effect of 14 different fungicides on the incidence of *Fusarium* fungi in winter wheat (*Triticum aestivum*) grain. The authors found that the fungicide containing a combination of active ingredients fenpropimorph, prochloraze and propiconazole decreased the incidence of *Fusarium* spp. in 75–100% of winter wheat kernels. Additionally, Sooväli et al. (2017) investigated the effect of barley seed treatment by fungicides containing various active ingredients. In greenhouse trials it was found that seed treatment before sowing of spring barley with different fungicide preparations containing tebuconazole alone, commercial mixtures of triticonazole and prochlorazole, fludioxonil and cyproconazole, fludioxonil and difenoconazole did not reduce the count of seed-borne inoculum of *Fusarium* spp. However, the active ingredients of triazole group of fungicides containing a combination of protioconazole and tebuconazole were most effective against the *Fusarium* fungi (Sooväli et al., 2017).

Studies conducted in Europe, Scandinavia and North America showed that the mycotoxin DON was present in 58–91%, mycotoxin T-2 in 50–61% and its deacetylated form mycotoxin HT-2 in 12–50% of barley grain samples. Thus, the mycotoxin DON is the most common toxin in barley samples (Petterson, 1996; Perkowski et al., 2003). Fungicide treatments to protect barley against *Fusarium* spp. and reduce mycotoxin accumulation in field conditions have resulted in controversial outcomes. In the Baltic region, attempts to control infestation of spring barley grains by mycotoxins have been carried out in Lithuania in a two-year study (Semaškiene et al., 2006), but the experiments were conducted in relatively warm and dry conditions, and there is no information about the efficacy of key fungicides in cooler and more humid climates further north. The aim of the present study was to investigate the impact of fungicide treatment: 1) on the yield and 1 000 kernel weight of spring barley, 2) on the incidence of *Fusarium* spp., to identify the effect of pure and mixed active ingredients of commercial fungicides on the production of the toxins DON, HT-2 and T-2 in the spring barley grain in field experiments. Additionally, the results of the current study allow to provide practical recommendations for farmers to reduce *Fusarium* spp. infection and mycotoxins infestation of barley grains.

2 Materials and methods

The field trials were carried out in 2012–2014 at the Estonian Crop Research Institute experimental area in Kõbu (59°27´N, 24°63´E) in North-Estonia. The soil was a sandy loam Gleysol according to WRB classification. The soil chemical analysis was carried out in the Laboratory of Agrochemistry of Agricultural Research Centre. The soil was weakly acid (pH 5.6), with high organic carbon (3.3%) and total phosphorus (139 mg kg^{-1})content, medium calcium (2271 mg kg^{-1}), magnesium (86 mg kg^{-1}), copper (1.6 mg kg^{-1}) and boron (1.35 mg kg^{-1}) content, and low potassium (51 mg kg^{-1}) and manganese (57 mg kg^{-1}) content. The experimental area was ploughed each autumn. The field plot size was 25 m^2 and the experiments were randomized in four replications. Two row spring barley (*Hordeum vulgare* L.) Estonian cultivar 'Maali' was used with a seed sowing rate of 550 seeds per m^2. The plots were fertilized with a complex mineral fertilizer 15N-15P_2O_5-15K_2O-9S (amount 270 kg ha^{-1}, nitrogen 40 kg, phosphorus 18 kg, potassium 36 kg and sulphur 24 kg ha^{-1}) at sowing time. Ammonium nitrate (N 60 kg ha^{-1}) was added in the beginning of stem elongation (BBCH 30) by top-dressing. The preceding crop was spring wheat. The fungicide treatments were applied as follows: 1) untreated control;

2) treated with Folicur 1.0 l ha^{-1}, (active ingredient 250 g l^{-1} tebuconazole); 3) treated with Falcon Forte 1.0 l ha^{-1}, (active ingredients 53 g l^{-1} prothioconazole, 224 g l^{-1} spiroxamine, 148 g l^{-1} tebuconazole); 4) treated with Archer Top 400 EC 0.8 l ha^{-1}, (active ingredients 275 g l^{-1} fenpropidin and 125 g l^{-1} propiconazole). In each case, the fungicides were applied with 300 l ha^{-1} water. The treatments with fungicides were carried out at flowering time, BBCH 65. For weed control, herbicide MCPA (2-methyl-4-chlorophenoxyacetic acid) was applied at a dose 2.0 l ha^{-1} in 400 l ha^{-1} water. No other pesticides were used.

2.1 Yield and 1000 kernel weight

Mature spring barley crop was harvested by a combine from each trial plot. The yield of every plot was dried, sorted, weighed and the samples were taken for the analysis of dry matter and 1 000 kernel weight. The hectare yield, Y_a (kg ha^{-1}), was calculated as: $Y_a = S_p D_s / A_p / 100$ where S_p is the plot yield (kg), A_p is the plot area (ha), and D_s is the standard percentage of dry matter (86%).

2.2 Incidence of Fusarium spp.

1.5 kg grain samples were taken from each variant for analysis of the incidence of *Fusarium* spp. and mycotoxins DON, HT-2 and T-2 in barley grain. The samples were dried and cleaned from debris and small kernels using a sieve with mesh size of 2 mm. One hundred kernels from each sample were taken. The kernels were cleaned in 1% sodium hypochlorite, and rinsed twice with distilled water. After drying, the kernels were put in a Petri dish on the Czapek-Dox medium (35 g Czapek-Dox broth, 15 g agar, 1 ml dichloran, 1 ml tetracycline, and 1000 ml MQ water). The plates were held under a day-night cycle (8 hr light/16 hr dark) at room temperature (20°C) for seven days, and then the number of the kernels contaminated with *Fusarium* spp. was counted. The *Fusarium* isolates from contaminated kernels were cultured on the PDA (potato dextrose agar) in 90 mm Petri dishes. The second isolation was done after a week in PDA and CLA (carnation leaf-piece agar). After 14 days, the *Fusarium* species were determined using a light microscope Olympus BX 51 (magnitude 100x) according to Leslie and Summerell (2006).

2.3 DON, HT-2 and T-2 quantification

Gas chromatography mass-spectrometry (GC-MS, Agilent 7890A and Agilent 5975C) was used for the determination of mycotoxins DON, HT-2 and T-2. The mycotoxins DON, HT-2 and T-2 were analysed according to the trichothecene analysis method by Saastamoinen and Saloniemi (1997). The detection threshold for each mycotoxin was 21.0 ± 0.5 µg kg^{-1}. Three replicate injections were taken from each variant for mycotoxin analysis.

2.4 The weather conditions

The air temperature, sum of precipitation and day of rainfall data were recorded by the weather station of the field experiments in Saku (Table 1).

2.5 The statistical analyses

Two factorial ANOVA was used for 2012–2014 field experiments data evaluation. Because the results strongly varied between the years, we used Tukey-Kramer Honest Significant Difference (HSD) test separately in each trial year. Average yield, 1 000 kernel weight (four replicate plots per treatment), incidence of *Fusarium* spp. (the percentage of infected kernels, three replicates per treatment) and concentrations of mycotoxins DON, HT-2 and T-2 (three replicates per treatment) were calculated for each treatment and study year. The concentrations of toxins T2 and HT2 were summarized since toxin T2 is metabolized to toxin HT2, and co-occurs in the grains (Nathanail et al., 2015; Hjelkrem et al., 2018). In all statistical tests, the level of significance was $p<0.05$.

3 Results

3.1 The weather in experimental years

The weather conditions during the three years of study were extremely different (Table 1). During the 2012 growing season the weather was rainy and cool compared to the long term average weather conditions (Table 1). With a lot of precipitation in June (83.6 mm), July (128.0 mm) and August (103.0 mm), the total amount of precipitation was 42–47% higher than the long-term average (57,90 and 73 mm, respectively). Overall, the weather conditions in 2013 were hot and dry, but August, when barley matured, was very rainy (110.0 mm) (Table 1). In 2014, the air temperatures and the amount of precipitation varied each month. June was cool (12.5°C) and wet (81.4 mm). Thereafter, July was hot (19.0 °C) and dry (42.8 mm), August hot (16.5 °C) with normal precipitation (Table 1). The results of our study showed that compared to 2013 and 2014, the rainy weather and low temperatures during the growing period in 2012 (Table 1) favoured grain contamination with mycotoxins DON and HT2+T2 in all trial variants (Table 4). At the same time, the average barley grain infestation with *Fusarium* spp. was low (3.8%) (Table 3). In 2013, dry and warm growing season combined with rainy and warm weather during maturation (Table 1) was favourable for contamination with the mycotoxin DON in barley grain in all trial variants (Tabel 4) and average incidence of *Fusarium* spp. was 15.3% (Table 3). In the warmth and normal precipitation levels of 2014 (Table 1) we detected mycotoxins DON, HT2 and T2 in both untreat-

ed and tebuconazole variants of barley grain (Table 4) and the average incidence of *Fusarium* spp. in barley grain was 10.9% (Table 3).

3.2 The yield of spring barley and 1 000 kernel weight

The average three-year barley yield was 3 058 kg ha^{-1} (Table 2), but varied between growing years from 898 kg ha^{-1} in 2012 to 4 286 kg ha^{-1} in 2014 (p<0.05). The highest average yield was achieved by untreated control (3 224 kg ha^{-1}) and the lowest in plots treated with fenpropidin and protioconazole (2 971 kg ha^{-1}), but these differences between the average yields for three years were not significant due to large variability that resulted from the exceptionally low yield in 2012

Table 1 *The weather conditions in the Saku experimental area, North-Estonia in 2012–2014*

		Air Temperature (˚C)				Precipitation (mm)				Number of rain days		
Month	Decade	2012	2013	2014	Long-term average	2012	2013	2014	Long-term average	2012	2013	2014
May	I	8	10	5.3		20.6	0	22.2		4	0	6
	II	10.2	13.5	11.9		30.8	28.6	12.8		5	3	5
	III	11.8	14.7	14.4		5.8	33.8	8.6		3	8	5
		10.1	**12.8**	**10.7**	**9.7**	**57.2**	**62.4**	**43.6**	**49.0**	**12**	**11**	**16**
June	I	9.7	17.3	15.2		27.2	4.2	17.2		5	2	4
	II	13.2	14.1	11.7		18.0	23.4	30.2		4	2	8
	III	12.8	18.7	10.7		38.4	12.6	34.0		7	1	5
		11.9	**16.7**	**12.5**	**14.5**	**83.6**	**40.2**	**81.4**	**57.0**	**16**	**5**	**17**
July	I	18.1	16.8	17.2		16.8	23.2	34.8		2	3	5
	II	14.6	16.3	18		87.2	6.8	3.0		6	3	2
	III	17.9	18	21.6		23.6	11.6	5.0		6	3	3
		16.9	**17.1**	**19.0**	**16.3**	**128**	**41.6**	**42.8**	**90.0**	**14**	**9**	**10**
August	I	15.3	18.4	20.6		34.4	55	8.6		7	4	2
	II	14.1	16	16.4		26.4	49.8	8.0		2	7	9
	III	13.1	14.8	12.9		42.6	5.2	52.0		8	2	7
		14.1	**16.3**	**16.5**	**15.3**	**103.0**	**110.0**	**68.6**	**73.0**	**17**	**13**	**18**
Average/Total		13.2	15.7	14.7	14.0	372.0	254.0	236.0	269.0	59	38	61

Long-term average values refer to the time period 1980—2010.

Table 2 *The yield and 1000 kernel weight in spring barley in 2012—2014*

Treatment	2012	2013	2014	Mean
Yield, kg ha^{-1}				
Untreated	765^{Cb}	4167^{Ba}	4740^{Aa}	3224^{a}
Tebuconazole (Folicur, 1.0 l ha^{-1})	1097^{Ba}	4198^{Aa}	3848^{Aa}	3048^{a}
Protioconazole, tebuconazole, spiroxamine (Falcon Forte 1.0 l ha^{-1})	904^{Bab}	3738^{Aa}	4319^{Aa}	2987^{a}
Fenpropidin, propiconazole (Archer Top 0.8 l ha^{-1})	826^{Bab}	3853^{Aa}	4235^{Aa}	2971^{a}
Mean	898^{B}	3989^{A}	4286^{A}	
1000 kernel weight (g)				
Untreated	27.0^{Cb}	45.7^{Ab}	43.6^{Bb}	38.8^{a}
Tebuconazole (Folicur, 1.0 l ha^{-1})	28.8^{Ca}	47.9^{Aa}	44.9^{Ba}	40.5^{a}
Protioconazole, tebuconazole, spiroxamine (Falcon Forte 1.0 l ha^{-1})	29.5^{Ba}	46.1^{Ab}	45.5^{Aa}	40.4^{a}
Fenpropidin, propiconazole (Archer Top 0.8 l ha^{-1})	28.8^{Ca}	46.3^{Ab}	43.6^{Bb}	39.5^{a}
Mean	28.5^{C}	46.5^{A}	44.4^{B}	

The data were compared by ANOVA followed by HSD test. Different uppercase letters show statistically significant (p<0.05) *difference among study years within the treatments and different lowercase letters show significant differences among treatments within studed years.*

Table 3 *The incidence (%) of Fusarium spp. in barley kernels in 2012—2014*

Treatment	2012	2013	2014	Mean
Untreated	3.0^{Aa}	14.0^{Aa}	6.0^{Aa}	7.7^{b}
Tebuconazole (Folicur 1.0)	5.0^{Aa}	19.0^{Aa}	24.8^{Aa}	16.3^{a}
Prothioconazole, tebuconazole, spiroxamine (Falcon Forte 1.0)	4.0^{Aa}	24.1^{Aa}	8.0^{Aa}	12.0^{a}
Fenpropidin, propiconazole (Archer Top 0.8)	3.0^{Aa}	4.0^{Ab}	5.0^{Ab}	4.0^{c}
Mean	3.8^{B}	15.3^{A}	10.9^{AB}	

The data were compared by ANOVA followed by HSD test. Different uppercase letters show statistically significant ($p<0.05$) *differences among study years within the treatments and different lowercase letters show significant differences among treatments within studed years.*

Table 4 *Effects of fungicides and year on the DON, HT-2 and T-2 mycotoxins content µg kg^{-1} in barley kernels in 2012—2014*

	DON µg kg^{-1}		
Treatment	**2012**	**2013**	**2014**
Untreated	73.3^{a}	63.0^{c}	65.5^{a}
Tebuconazole (Folicur 1.0)	66.0^{b}	63.0^{bc}	64.8^{a}
Prothioconazole, tebuconazole, spiroxamine (Falcon Forte 1.0)	69.7^{ab}	63.1^{a}	0.0^{b}
Fenpropidin, propiconazole (Archer Top 0.8)	66.0^{b}	63.1ab	0.0^{b}
P value	*0.001*	*0.009*	*<0.001*
	HT-2 and T-2, µg kg^{-1}		
Untreated	27.5^{b}	0.0^{a}	32.9^{a}
Tebuconazole (Folicur 1.0)	26.2^{bc}	0.0^{a}	32.6^{a}
Prothioconazole, tebuconazole, spiroxamine (Falcon Forte 1.0)	25.6^{c}	0.0^{a}	0.0^{b}
Fenpropidin, propiconazole (Archer Top 0.8)	62.3^{a}	0.0^{a}	0.0^{b}
P value	*<0.001*	*ns*	*<0.001*

Factor	DON	HT-2 and T-2
Year	*0.001*	*<0.001*
Treatment	*ns*	*ns*
Year*Treatment	*<0.001*	*<0.001*

Different letters behind the mean values (n=3) indicate significant differences ($p<0.05$) *in a category.*

(Table 2). The highest barley yield compared to the untreated control, 1 097 kg ha^{-1}, was obtained in 2012 in tebuconazole treated plots ($p<0.05$) (Table 2). No differences in yield among other treatments were observed in 2012 and 2013 (Table 2). The highest yield of barley was obtained in 2014, however the yield in control and treated plots was not significantly different. The overall average 1 000 kernel weight was 39.8 grams, lowest ($p<0.05$) in 2012 (28.5 grams) and highest in 2013, (46.5 grams) (Table 2). The highest average 1 000 kernel weight in the three years was found in the variant of tebuconazole (40.5 g) and lowest in the variant of untreated control (38.8 g). As the three-year average results among treatments were not significantly different, the treatment with fungicides had no effect on the 1 000 kernel weight (Table 2).

3.3 Incidence of Fusarium spp. and mycotoxins DON, HT-2 and T-2

The three-year incidence of *Fusarium* spp. was on average 10.0% (Table 3). The impact of year together with fungicide treatment had a significant effect on the incidence of *Fusarium* spp. ($p<0.05$) (Table 3) and on the incidence of mycotoxins (Table 4) in barley kernels ($p<0.001$).The incidence of *Fusarium* spp. in barley grain was lowest in 2012 and highest in 2013 (Table 3). In 2012, *Fusarium* spp. was present on average in 3.8% of grains (Table 3). In 2013, the incidence of *Fusarium* spp. was on average 15.3%, whereas 14.0% of barley grain in the untreated variant was contaminated with *Fusarium* fungi (Table 3). The incidence of *Fusarium* spp. in the variants with tebuconazole or a commercial mixture of three active ingredients (prothioconazole, tebuconazole, spiroxamine) was 19.0% and 24.1%, respectively. The lowest incidence

of *Fusarium* spp. of only 4% of barley kernels, was found in the commercial mix of active ingredients phenpropidin and propiconazole (Table 3). In 2013 and 2014, spraying with fungicides reduced (p<0.05) the incidence of *Fusarium* spp. in grains from plants treated with the commercial mix of active ingredients phenpropidin and propiconazole variants, where the incidence of *Fusarium* spp. was only 4% and 5%, respectively (Table 3). The concentration of mycotoxins DON, HT-2 and T-2 in barley kernels was low during the study. The year effects varied for different treatments for different mycotoxins (Table 4). Mycotoxin DON was detected in all trial variants of barley in 2012 and 2013 and in untreated and tebuconazole variants in 2014. HT2+T2 toxins were present in all variants in 2012 and in the untreated and tebuconazole variants in 2014 (Table 4). In 2012, concentration of the mycotoxin DON was highest (p=0.001) in the untreated variant (73.3 μg kg^{-1} barley grain), compared with the treated variants (Table 4). Higher DON levels were also present in barley kernels treated by the commercial mix of three active ingredients prothioconazole, tebuconazole and spiroxamine (69.7 μg kg^{-1}) compared to barley from the tebuconazole or the commercial mix of fenpropidin and propiconazole variants. In 2012, DON concentrations were significantly lower (p=0.001) in the variants with tebuconazole and with a commercial mix of active ingredients fenpropidin and propiconazole (Table 4). In 2012, mycotoxins HT2+T2 were detected in all trial variants and their concentration was significantly higher (62.3 μg kg^{-1}) (p=0.001) in the variant with the commercial mix of active ingredients fenpropidin and propiconazole (Table 4). The lowest HT2+T2 content (25.6 μg kg^{-1}) (p<0.05) was found in barley variant treated by the commercial mixture of three active ingredients (prothioconazole, tebuconazole and spiroxamine) (Table 4).

In 2013 the mycotoxin DON was detected in all trial variants of barley (Table 4). The highest DON content, 63.1 μg kg^{-1}, was found in barley treated with commercial mix of three active ingredients protioconazole, tebuconazole, spiroxamine and the lowest in barley from untreated variant (63.0 μg kg^{-1}) (p=0.009) (Table 4). In 2013, mycotoxins HT2+T2 were not detected in barley (Table 4).

In 2014 the mycotoxin DON was present only in untreated (65.5 μg kg^{-1}) and tebuconazole-sprayed (64.8 μg kg^{-1}) variants (p<0.001) (Table 4). Mycotoxins HT2+T2 were similarly detected only in the untreated (32.9 μg g^{-1}) and tebuconazole (32.6 μg kg^{-1}) variants, but not in the barley treated by the commercial mix of three active ingredients prothioconazole, tebuconazole and spiroxamine or by the commercial mix of the active ingredients fenpropidin and propiconazole (p<0.001) (Table 4).

4 Discussion

In a three-year field experiment,we studied the effect of fungicide application at flowering time in spring barley on the yield, 1000 kernel weight, incidence of *Fusarium* fungi and mycotoxins DON, HT-2 and T-2. The results of our study showed that the yield and 1000 kernel weight of spring barley were similar in most fungicide treatments and in untreated control in years with a high barley yield, but not in the year with a low yield. Also, the spraying with fungicides had not a clear effect on the barley yield and 1000 kernel weight, and primarily the study year was the main factor that affected barley yield and 1000 kernel weight. As in our study, Stetkiewicz et al., (2019) also concluded that application of fungicide had no effect on barley yield. From the long-term field trials, the application of fungicides resulted in a significant yield increase in only 35% of cases (Stetkiewicz et al., 2019). On the other hand, in other field trials with spring barley it was shown that the impact of year had a stronger influence on the plant diseases; and spraying of more resistant varieties with fungicides at late growth stage decreased the yield (Sooväli and Koppel, 2009). In field trials designed to compare single to double application of fungicides, the highest yield and 1000 kernel weight were achieved after a double fungicide application (Caldwell et al., 2017). In our trials the barley heads were treated with fungicide only once at the flowering time, and the non-significant effect of fungicides in high-yield years might indicate that one treatment was not enough to get the highest yield and 1000 kernel weight.

In our study, differences in weather among different study years had a stronger influence on the incidence of *Fusarium* spp. on kernels compared to the effect of fungicides. The effect of treatment with fungicides varied between the years, and only the fungicide with two active ingredients (fenpropidin and propiconazole) was found to decrease the incidence of *Fusarium* spp. in barley. Analogous equivocal results have been observed in other studies. In Lithuanian field trials, the commercial mixture of protioconazole and tebuconazole effectively decreased *Fusarium* spp. contamination in barley kernels (Semaškiene et al. 2006). In addition, in accordance with our results, it turned out that the weather had a strong impact to the efficiency of fungicides on *Fusarium* spp. (Semaškiene et al., 2006). Unlike our study and that of Semaškiene et al. (2006), several other studies have demonstrated that single fungicides, e.g. tebuconazole, are effective in controlling *Fusarium* incidence. The active ingredients of fungicides may also have a positive impact on the incidence of *Fusarium* spp. in cereal grain (Gaurilčikienė et al., 2011). We did not find a similar eff-

fect. However, the results of two study years showed that for tebuconazole and for the commercial mix of prothioconazole, tebuconazole and spiroxamine the incidence of *Fusarium* spp. was higher in the barley grain compared to samples that were untreated or treated with the commercial mix containing fenpropidin and propiconazole. Some studies found that various ingredients of fungicides may support the production of trichothecenes by *Fusarium* spp. in wheat (Giraud et al., 2011), also in rye and triticale kernels (Gaurilčikienė et al., 2011). In our study the levels of mycotoxins DON, HT-2 and T-2 in barley kernels varied from year to year and the application of the fungicides showed a variable effect. Mycotoxin DON was detected in barley kernels in all years, but it occurred only in untreated and tebuconazole-treated variants in 2014. Mycotoxins HT2 + T2 were found in barley grains in all variants in 2012, but only in untreated and tebuconazole-treated variant in 2014. The weather conditions have a strong impact on the incidence of mycotoxins in barely grain. Although the trend showed that the treatment with fungicides reduced the mycotoxin DON in barley grain, the effect of active ingredients on mycotoxin DON in barley was not clear. The efficiency of fungicides in decreasing mycotoxin HT2 and T2 in barley grain was not demonstrated. Similarly, the field trials conducted in the Czech Republic over four years with spring barley revealed, that the mycotoxin content in kernels varied between the years, but the combination of the active fungicide ingredients decreased the accumulation of DON (Váňova et al., 2004). In France, it was also found that in naturally infected conditions in winter barley during three experimental years the average DON content was very low (<20 μg kg^{-1}) and the fungicide treatment had an indistinct effect on *Fusarium* infection (Ioos et al., 2005). In our study, the concentration of mycotoxins was also low. Ioos et al., (2005) concluded that in the first experimental year, better effect was achieved using a complex fungicide containing a mixture of active ingredients. In the second year, six single ingredient fungicides had better effect and in the third experimental year, only one fungicide was effective against *Fusarium* spp. Moreover, the treatment with fungicides had no effect on the accumulation of DON and NIV (Ioos et al., 2005). The study of Malachova et al., (2010) with several varieties of brewery barley found that 86% of samples were contaminated with DON and 62% of samples with HT-2. Nevertheless, weather had the strongest impact on the occurrence of mycotoxins (Malachova et al., 2010). Běláková et al., (2014), based on a four-year study with malting barley, also concluded, that the weather influenced the contamination of kernels with mycotoxins. The results of our experiments in field conditions confirm that fungicides were not clearly effective in reducing the content of mycotoxins DON, HT-2 and T-2 in barley grain, because the impact of weather was stronger. Many researchers have found that weather conditions during heading, flowering and ripening time of cereals affected the incidence of *Fusarium* and mycotoxins in cereal kernels; heavy rainfall during these growth stages favoured the incidence of *Fusarium* spp. and mycotoxins in grain (Mankevičiene et al., 2011). In the Estonian climate, the flowering, development of kernels and ripening of spring cereals occur from July to the beginning of September. In this study the weather conditions of heading, flowering and ripening stages varied year by year. However, it is in these growth stages that the *Fusarium* spp. infected the heads of cereals and started to produce mycotoxins (Osborne and Stein, 2007; Burlakoti et al., 2011). Edwards (2009) found that weather conditions during the growing season influenced significantly the contamination of barley grain with mycotoxins. The occurrence of different mycotoxins such as DON, 3-ADON, 15-ADON, HT-2, T-2 and fusarenoon X, varied between the years (Edwards, 2009). The concentrations of the mycotoxin DON in our study were lower than the maximal limits allowed by the European Commission legislation (1 250 μg kg^{-1}, EC 1881/2006). In earlier research it was declared that the mycotoxin DON occurred most frequently in barley grain in Europe, Scandinavian and North-America, being found in 58–91% of samples, mycotoxin HT-2 was found in 12–50% of barley samples and mycotoxin T-2 was detected in 58–91% of barley samples. The average concentration of DON in Europe is 189 μg kg^{-1} and in Scandinavian barley it is 229 μg kg^{-1} (Petterson, 1996; Perkowski et al., 2003).

5 Conclusions

A single treatment of spring barley crop with fungicide at flowering time had no effects on the yield and 1000 kernel weight. Three-year average results showed that the effectiveness of fungicides to reduce *Fusarium* fungi and to prevent the grain contamination with mycotoxins varied from year to year. The treatment of spring barley with a fungicide containing a commercial mix of active ingredients fenpropidin and propioconazole decreased the incidence of *Fusarium* spp. in grain. The content of mycotoxins DON, HT2 and T2 in barley grain was influenced by the interactions of the weather during the growing season and depended on the active ingredients of the fungicides. Hence, we suggest that the use of fungicides is not economically viable to decrease the content of mycotoxins in grains.

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Production of fusarielins by *Fusarium*

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ABSTRACT

Fusarielins constitute a relative unexplored group of secondary metabolites, which have been isolated mainly from unidentified *Aspergillus* and *Fusarium* strains. In the present study we show that the ability to produce fusarielins is restricted to a few *Fusarium* species. Among the 15 analyzed species fusarielins were identified only in extracts from *Fusarium graminearum* and *Fusarium tricinctum*. The influence of different carbon sources on fusarielin biosynthesis was examined and the results showed that disaccharides and dextrin in combination with arginine as sole nitrogen source increased fusarielin production. When arginine was replaced with nitrate the fusarielins were produced on a wider selection of carbon sources including all monosaccharides. Production of fusarielins in *F. graminearum* was also influenced by pH, cultivation time, temperature and fructose concentration with the optimal conditions being: pH 6, 25 °C, 26 days and 60 mg fructose/mL. Wheat spikes were inoculated with *F. graminearum* to determine whether fusarielins are produced in infected cereals and fusarielin H was detected in all samples ranging from 392 to 1865 ng/g (mean: 989 ng/g) indicating that fusarielins are produced during infection. The study shows that even though fusarielins are produced by a narrow list of *Fusarium* species, they potentially can be present in infected cereals.

1. Introduction

The genus *Fusarium* comprises more than 70 species, which occur in many habitats and produce a wide array of bioactive secondary metabolites (Summerell and Leslie, 2011). Some of these compounds are produced by a large number of species while others are limited to a few or only a single species (Thrane, 2001). One metabolite group, which has received little attention in the *Fusarium* community, is the fusarielins. The ability of the *Fusarium* species to produce fusarielins is largely unknown. Fusarielins A–E were first isolated and characterized from unidentified *Fusarium* strains (Kobayashi et al., 1995; Namikoshi et al., 2000; Gai et al., 2007) and later fusarielins A and B were isolated from a *F. tricinctum* strain (Nenkep et al., 2010). Recently we added three fusarielin analogues F, G and H to the list of known metabolites from *Fusarium* by ectopic activation of the responsible polyketide synthase (PKS) gene cluster in *F. graminearum* (Sørensen et al., 2012). The gene cluster is not present in the sequenced *Fusarium oxysporum*, *Fusarium solani* and *F. verticillioides* strains (Hansen et al., 2012), indicating that the ability to produce fusarielins is not conserved throughout the *Fusarium* genus. The fusarielin gene cluster with the polyketide synthase, transcription factor and tailoring enzymes is also present in *Aspergillus clavatus*, *A. fumigatus* and *Metarhizium anisopliae* with some rearrangements compared to *F. graminearum* (Sørensen et al., 2012). These gene clusters are likely to be functional since fusarielins A, B and E have been isolated from an unidentified *Aspergillus* strain (Nguyen et al., 2007) and eight additional fusarielin analogues (ICM0301 A–H) have been isolated from another *Aspergillus* strain (Someno et al., 2004).

Fusarielins have been shown to interfere with the microtubule function in the ascomycete *Pyricularia oryzae* and cause mycelia deformations (Kobayashi et al., 1995; Gai et al., 2007). In antibacterial assays fusarielins A, B and E were found to be mildly antibiotic with fusarielin A as the most potent (Nguyen et al., 2007). Fusarielins have also been shown to have toxic effect on human epithelial carcinoma cell lines (Kobayashi et al., 1995; Sørensen et al., 2012). We have found that fusarielins A, F, G and H can be characterized as mycoestrogens as they stimulate growth of MCF-7 breast cancer cells (Sondergaard et al., 2012) in a similar manner as another *Fusarium* metabolite, fusarin C (Sondergaard et al., 2011). To gain an insight into the occurrence of fusarielins in the environment we examined a broad selection of *Fusarium* species that infect our crop plants. In addition the effects of variable carbohydrate sources, pH, temperature and time were examined to determine which external factors influence fusarielin production (Fig. 1).

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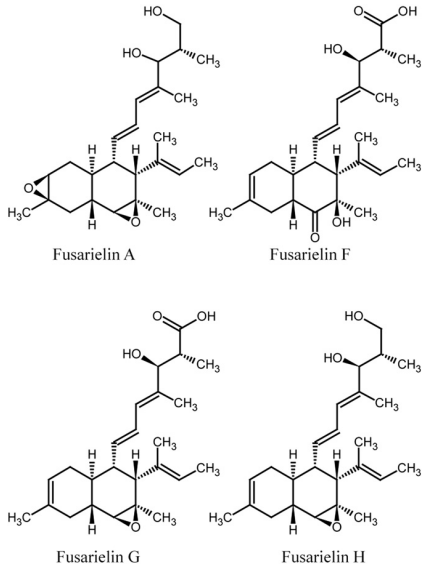

Fig. 1. Structures of fusarielins A, F, G and H.

2. Materials and methods

2.1. Chemicals and standards

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) and carbohydrates were purchased from Sigma-Aldrich and Merck (Whitehouse Station, NJ, USA). Standards of fusarielins A, F, G and H were available from previous studies (Kobayashi et al., 1995; Sørensen et al., 2012) and kept in methanol in individual 1 mg/mL stock solutions at −20 °C.

2.2. Detection of fusarielins

Initial screens for the presence of fusarielins A, F, G and H in the selected *Fusarium* species was performed by LC–MS by using a Bruker Daltonics micrOTOF II (Bremen, Germany) operating in the positive mode scanning 50–1000 amu. For the quantification of fusarielins A, F, G and H all samples were analyzed on a Dionex UltiMate 3000 UHPLC system (Idstein, Germany) connected to a Thermo Vantage triple stage quadrupole mass spectrometer (Thermo Fisher Scientific, San José, CA, USA) with a heated electrospray ionization probe. Five μL extract was injected and separated on a Gemini C6-Phenyl 3 μm 2-mm i.d.×50-mm column (Phenomenex, Torrance, CA, USA) by using a constant flow of a 0.3 mL/min and acetonitrile–water gradient both containing 0.1% formic acid. The gradient started at 25% acetonitrile, increasing to 100% over 10 min, which was maintained for 1 min before reverting to 25% acetonitrile in 1 min and recalibrated for 2 min. The following ion source parameters were used for the detection of fusarielins: spray voltage (4.5 kV), vaporizer temperature (350 °C), nitrogen sheath gas pressure (30 arbitrary units), nitrogen auxiliary gas pressure (10 arbitrary units), and capillary temperature (270 °C). Argon was used as the collision gas and set to 1.5 mTorr. A Gemini C6-Phenyl column was used for chromatographic separation as this column increased retention compared to several C18 columns that were initially tested. The C6-Phenyl column has also previously been shown to increase retention of other *Fusarium* metabolites (Sørensen et al., 2008). The MS/MS settings for the individual fusarielins are listed in Table 1. A twofold dilution series (9.8–10,000 ng/mL) of fusarielins A, F, G and H was made and the compounds were detected in a linear fashion in the dilution series with R^2 values for fusarielins A, F, G and H of 0.985, 0.987, 0.991 and 0.993 respectively.

2.3. Production of fusarielins by Fusarium species

Representative strains of fifteen different *Fusarium* species, *F. avenaceum* (IBT 40847, 8464, and 2950), *F. cerealis* (IBT 40125, −40126, and −40850), *F. culmorum* (IBT 9614, −2925, and −8099), *F. equiseti* (IBT 8752 and −40221), *F. graminearum* (NRRL 31084 and IBT 1955 and −9203), *F. langsethiae* (IBT 9955, −9959, and −8051), *F. oxysporum* (IBT8890, −9065, and −9805), *F. poae* (IBT 9928, −9999, and −9982), *F. proliferatum* (IBT 41107, −40647, and −40643), *F. pseudograminearum* (NRRL 28069 and IBT 1547 and −1544), *F. sambucinum* (IBT 2524, −1731, and −2364), *F. solani* (IBT 2970 and −6161), *F. sporotrichioides* (IBT 9948 and −1926), *F. tricinctum* (IBT 2952, −8166, and −40088) and *F. venenatum* (IBT 1197, −1337, and −2203), were selected from the IBT collection at the Technical University of Denmark and from the Agricultural Research Service culture collection (NRRL) at the National Center for Agricultural Utilization Research in Peoria, Illinois, USA (origin listed in Supplementary Table 1) to examine their ability to produce fusarielins F, G and H in a single replicate screening experiment. The *Fusarium* strains were grown for one week on Czapek Dox agar plates (CZ; Difco Sparks, MD, USA) and mycelium was transferred by a three point inoculation onto 90 mm Petri dishes containing malt extract agar (MEA; Merck), oat meal agar (OA), potato dextrose agar (PDA; Merck), yeast extract sucrose agar (YES) or CZ (Samson et al., 2010) and incubated for two weeks in the dark at 25 °C.

2.4. The effect of growth factors on fusarielin production

F. graminearum (IBT 9203) was selected as test strain to examine the effect of growth factors on fusarielin production. Spores were produced by growing the strain in a 250 mL baffled flask containing 50 mL liquid sporulation medium (Yoder and Christianson, 1998) for three days at 20 °C in the dark at 150 rpm. The spores were isolated by centrifugation and dissolved in sterile H_2O to give a final concentration of 1×10^6 spores per mL. Twenty μL spore suspension was inoculated to 14 mL tubes containing 1 mL of liquid modified Czapek Dox (CZ) medium (pH 6) containing (five replicates): 30 mg carbon source; 3 mg $NaNO_3$; 1 mg K_2HPO_4; 0.5 mg KCl; 0.5 mg $MgSO_4\cdot7H_2O$; 0.01 mg $FeSO_4\cdot7H_2O$ and 1 μL trace metal solution (1 g $ZnSO_4\cdot7H_2O$ and 0.5 g $CuSO_4\cdot5H_2O$ in 100 mL H_2O) and grown for two weeks at 25 °C in the dark. The different carbon sources were two aldopentoses (arabinose and xylose), two ketohexoses (fructose and sorbose), three aldohexoses (galactose, mannose and glucose), four disaccharides (lactose, maltose, sucrose and trehalose)

Table 1
Parameters for selected reaction monitoring (SRM) of fusarielins.

	RT (min)[a]	Precursor ion (m/z)	Product ions (m/z)[b]	S-lens (V)	CID (V)[c]
Fusarielin F	4.33	415.2 $[M-H]^-$	73.1/341.2	91	22/16
Fusarielin A	4.45	385.3 $[M+H-H_2O]^+$	99.0/165.1	70	17/80
Fusarielin G	5.63	399.2 $[M-H]^-$	73.1/355.6	89	22/20
Fusarielin H	5.79	369.2 $[M+H-H_2O]^+$	105.1/91.1	72	42/51

[a] Retention time.
[b] Quantifier/qualifier ions.
[c] Collision induced dissociation energy for quantifier/qualifier ions.

and six polysaccharides (dextrin, glycogen, and xylan and starch from corn, rice and potato). The carbohydrates were added to the autoclaved medium after cooling in order to prevent degradation and the experiment was performed with arginine as a nitrogen source for *F. tricinctum* and arginine or nitrate for *F. graminearum*.

CZ with fructose (30 mg/mL) as carbon source and $NaNO_3$ (3 g/L) as a nitrogen source was used to examine the effects of varying pHs (4, 5, 6, 7, 8 and 9 ± 0.1), temperature (15, 20, 25, 30 and 37 °C), fructose (0, 15, 30, 60, 120, and 240 mg/mL) and time (6, 10, 14, 18, 22 and 26 days) on fusarielin production by using the same procedure as described above (five replicates).

2.5. Infection of wheat spikes

Seven wheat spikes were point-inoculated at anthesis with spores of *F. graminearum* strain NRRL 31084 (Josefsen et al., 2012). The wheat spikes were harvested 34 days after inoculation and stored at −20 °C until their analysis.

2.6. Extraction of fusarielins

Extraction of solid cultures was adapted from the micro scale method previously described (Smedsgaard, 1997). In brief, six plugs (6 mm) from the two week old cultures were first extracted ultrasonically for 30 min with 1 mL ethyl acetate and successively with 1 mL ethyl acetate: dichloromethane:methanol (3:2:1) both containing 1% formic acid. The pooled extracts were evaporated to dryness in vacuo and re-dissolved ultrasonically in 600 mL methanol. Liquid cultures (1 mL) were extracted with 3 mL acetonitrile/water/acetic acid (79/20/1) by using a Vibracell VC130 sonicator (Sonics & Materials, Inc., Newtown, CT, USA) with an amplitude of 100 for 10 s per sample subsequently by rotation at 180 rpm for 1.5 h. 1 mL extract was transferred to a 2 mL eppendorf tube, spun for 2 min at 12,000 rpm (revolutions per minute) in a centrifuge and then transferred to a 2 mL HPLC vial.

The wheat spikes were ground to a fine powder with liquid nitrogen prior to the extraction of metabolites. The samples were weighed and 20 mL acetonitrile:H_2O (85:15) per g wheat spike was added and extraction was carried out for 1 h at 200 rpm. Extracts from wheat spikes (1 mL) and solid cultures (600 μL) were spun for 2 min at 12,000 rpm in 2 mL eppendorf tubes and then transferred to 2 mL HPLC vials for analysis.

2.7. Bioinformatics

Information on conservation across species and expression profiles for the fusarielin polyketide synthase *FSL1* gene cluster was extracted from public databases: MAS 5.0 normalized data from cross species hybridization (FG3) (Güldener et al., 2006), expression during crown rot of wheat (FG12) (Stephens et al., 2008), wheat head blight (FG15) (Lysøe et al., 2011), and infection of wheat stems (FG16) (Guenther et al., 2009) obtained from the plant expression database (plexdb.org) (Dash et al., 2012) was examined.

3. Results and discussion

3.1. Quantification of fusarielins

To enable the quantification of fusarielins A, F, G and H in fungal extracts and infected wheat spikes we developed an LC–MS/MS method. The MS/MS parameters and settings for each compound were automatically adjusted to obtain the optimal quantification conditions (Table 1 and Fig. 2). Fusarielins F and G quantification was optimal when the MS operated in the negative mode, possibly due to the presence of the carboxyl acid group. All four fusarielins showed a high degree of fragmentation, but because of their strong ionization properties they could easily be detected at the lowest concentration (9.8 ng/mL).

3.2. Production of fusarielins by Fusarium species

The distribution of the ability to produce fusarielins was examined by using a selection of strains from fifteen different species. All strains were grown on five diverse media to examine possible regulation patterns and increase the chance of activating silent fusarielin gene clusters. The results (Table 2) showed that fusarielin A was only produced by *F. tricinctum* and that fusarielins F, G and H were only produced by *F. graminearum*. None of the fusarielins could be detected in extracts from *F. avenaceum*, *F. cerealis*, *F. culmorum*, *F. equiseti*, *F. langsethiae*, *F. oxysporum*, *F. poae*, *F. proliferatum*, *F. pseudograminearum*, *F. sambucinum*, *F. solani*, *F. sporotrichioides* and *F. venenatum* suggesting that the ability to produce fusarielins is limited to a few *Fusarium* species. It is possible that some species produce novel fusarielins which were not detected in our present chemical analyses, or that some fusarielin gene clusters are silent under the tested conditions. Cross species hybridization data (FG3) from the Plant Expression Database (Dash et al., 2012) showed that the signal was from two other members of the *F. graminearum* species clade, *F. boothii* and *F. asiaticum* (Supplementary Fig. 1). The signal from *F. culmorum* and *F. pseudograminearum*, two species very closely related to *F. graminearum*, was comparable to *F. oxysporum* and *F. verticillioides*, where the genome sequence revealed the absence of the fusarielin gene cluster (Hansen et al., 2012).

Similarly fusarielins were not produced by *F. avenaceum* although it is closely related to *F. tricinctum* (Yli-Mattila et al., 2002) and shares the ability to produce several secondary metabolites including enniatins and moniliformin (Langseth et al., 1999).

The results showed that the production of fusarielins in different strains of *F. tricinctum* varied with respect to substrate preference and quantity. Two strains (IBT 2952 and 8166) had the highest fusarielin A production on CZ medium followed by YES. The third strain (IBT 40088) produced lowest amounts of fusarielin A on CZ and highest amounts on YES. MEA was the least inducive medium for the production of fusarielin A by any of the three strains.

The three *F. graminearum* strains all produced very high amounts of fusarielins on YES medium compared with the other media. Fusarielin H was the dominant analogue followed by G and F as also observed previously (Sørensen et al., 2012). Strain IBT 9203 produced the highest concentrations of fusarielin H on YES (77.0 μg/mL extract), which is >23 times higher than the second best medium, MEA. The genome sequenced strain (NRRL 31084) is generally a low producer of secondary metabolites and produced also the lowest amount of fusarielin H on the five media tested. As this strain has been used as a model in many studies it may explain why fusarielins have remained undiscovered in

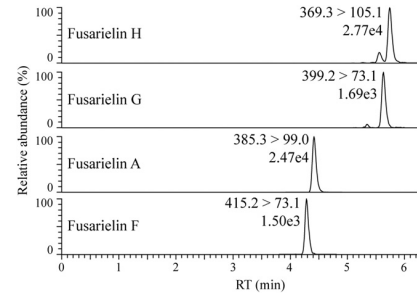

Fig. 2. LC–MS/MS detection of quantification traces for fusarielins A, F, G and H (156 ng/mL).

Table 2
Production of fusarielins A and H (μg/mL) by *Fusarium graminearum* and *F. tricinctum* species grown on Czapek Dox agar (CZ), malt extract agar (MEA), oat meal agar (OA), potato dextrose agar (PDA) and yeast extract sucrose agar (YES)[a].

Species	Collection	Fusarielin A					Fusarielin H				
		CZ	MEA	OA	PDA	YES	CZ	MEA	OA	PDA	YES
F. graminearum	NRRL 31084	nd	nd	nd	nd	nd	Trace	Trace	Trace	Trace	10.3
F. graminearum	IBT 1955	nd	nd	nd	nd	nd	1.30	0.30	Trace	Trace	36.9
F. graminearum	IBT 9203	nd	nd	nd	nd	nd	0.44	3.24	0.23	0.74	77.0
F. tricinctum	IBT 2952	33.7	0.31	0.98	1.27	5.39	nd	nd	nd	nd	nd
F. tricinctum	IBT 8166	27.0	0.16	1.21	1.19	1.75	nd	nd	nd	nd	nd
F. tricinctum	IBT 40088	Trace	0.65	2.04	1.36	2.21	nd	nd	nd	nd	nd

[a] nd: not detected, trace: <0.01 μg/mL.

F. graminearum until the gene cluster was up-regulated by molecular manipulation (Sørensen et al., 2012).

3.3. Influence of carbohydrates on fusarielin production

The effect of carbon source on fusarielin production was examined by growing *F. graminearum* and *F. tricinctum* strains (IBT 9203 and IBT 2952) on 18 different carbohydrates. The results showed that *F. tricinctum* produced highest amounts of fusarielin A when grown on the more complex carbon sources. Dextrin resulted in the highest concentration of fusarielins in *F. tricinctum* extracts, 2.6 μg/mL, followed by maltose, sucrose, xylan and trehalose. Fusarielin A was produced on all monosaccharides with arabinose resulting in the highest concentration (264 ng/mL) and fructose the lowest (21 ng/mL).

In *F. graminearum* the production of fusarielins was regulated by both nitrogen source and carbon source as seen in Fig. 3. The amounts of the different fusarielins produced were again in the order H > G > F. Fusarielin H was generally produced on a wider selection of carbon sources when nitrate was used instead of arginine. When nitrate was used the highest production of fusarielin H was obtained on cellobiose (1.5 μg/mL) followed by fructose, arabinose and maltose. When arginine was used as a nitrogen source the highest production of fusarielin H was obtained on arabinose (1.4 μg/mL) followed by lactose, cellobiose and trehalose. Fusarielin H was produced in detectable amounts on all monosaccharides in combination with nitrate, but only on arabinose when arginine was used.

The results suggest that there is a complex regulation of fusarielin production which is influenced by nitrogen and carbon sources. Nitrogen source has a strong influence on other secondary metabolites produced by *F. graminearum* such as deoxynivalenol (DON), which is induced by arginine and repressed by nitrate with sucrose as the carbon source (Gardiner et al., 2009). In the same study it was found that the repressive effect of nitrate on DON production could not be overcome by any of the 190 carbon sources tested. Jiao and co-workers used glutamic acid as nitrogen source and found that carbon sources also influenced DON production (Jiao et al., 2008). The production of the red pigment aurofusarin has been shown in *F. graminearum* to be influenced by both nitrogen and carbon sources (Zhang and Wolf-Hall, 2010). These observations comply with the findings that the nitrogen regulator *AreA* and the carbon regulator *creA* have a strong regulatory influence on the biosynthesis of secondary metabolites in filamentous fungi (Yu and Keller, 2005).

3.4. Production of fusarielins in response to abiotic factors

The effect of time, pH, temperature and fructose on fusarielin production was examined by cultivating *F. graminearum* (IBT 9203) under different conditions. The medium chosen contained nitrates combined with different amounts of fructose as this was found to enhance fusarielin production (Fig. 3). The production of fusarielins F, G and H generally followed the same pattern and again the amounts were in the order H > G > F as seen in Fig. 4. The fungus was unable to grow on medium without fructose and the highest concentration of fusarielins was observed at 60 mg/mL fructose. At the highest fructose concentration (240 mg/mL) the fungus grew well but the production of fusarielins was almost completely inhibited.

The optimal temperature for the production of fusarielins was 25 °C, as a production of fusarielin H was 88 and 86% lower at 20 °C and 30 °C, respectively. For the varying pH conditions tested the highest production of fusarielins occurred at pHs 5 and 6 and was lower although constant at higher pH (7–9). In the time study the production of fusarielins F, G and H increased generally with time and the maximum amount of fusarielins G and H was observed in the oldest cultures after 18 and 26 days, respectively.

The repressive effect of fructose on fusarielin production could either be due to a direct response to the high fructose content or a response to the changes in osmotic conditions as adding 240 mg/mL fructose to the medium will reduce water activity to approximately 0.97–0.98 (Ben Gaida et al., 2006). Osmotic conditions have previously been shown to have an effect on the production of zearalenone and DON in *F. graminearum* (Hope et al., 2005; Kokkonen et al., 2010). Fumonisins, another group of *Fusarium* metabolites, were also affected by changes in osmotic conditions induced by glucose, NaCl and glycerol (Mogensen et al., 2009).

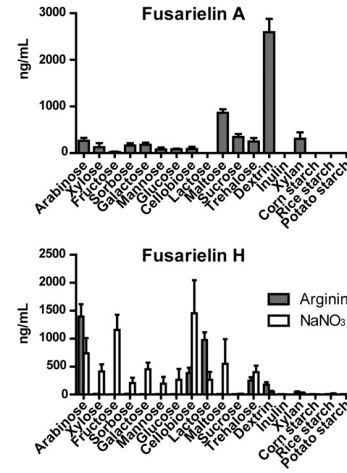

Fig. 3. Production of fusarielins A and H in response to different carbohydrates by *F. tricinctum* (IBT 2952, top) and *F. graminearum* (IBT 9203, bottom). For *F. tricinctum* the nitrogen source was arginine whereas experiments with *F. graminearum* included arginine and nitrate. The bars represent means of three (nitrate) or five (arginine) replicates (±SEM).

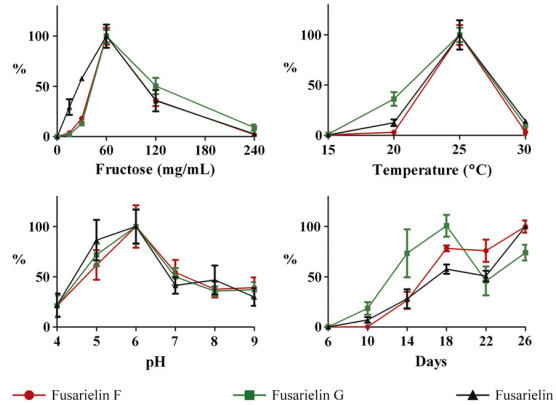

Fig. 4. Mean ± SEM production of fusarielins F, G and H by *F. graminearum* (IBT 9203) under variable conditions, displayed as relative abundance % with the highest mean of each analogue set to 100% in each experiment (five replicates). In the experiments with fructose, temperature, pH and time 100% were 2.74; 0.48; 1.58; 0.39 μg/mL for fusarielin F, 13.4; 1.82; 1.73; 1.27 μg/mL for fusarielin G and 13.8; 4.15; 17.5 and 3.29 μg/mL for fusarielin H.

In our study we observed optimal production of fusarielins at 25 °C and in the study by Hope et al. (2005) temperature has also been shown to influence the production of DON, with 25 °C resulting in a higher production than 15 °C, which was also observed for zearalenone by Kokkonen et al. (2010).

Our results suggest that fusarielins are mildly regulated by an ambient pH with an optimum at pHs 5–6. An influence of pH has also been observed for zearalenone, where acidic conditions induce the responsible gene cluster (Kim et al., 2005). The regulation of secondary metabolites in response to acidic and basic conditions can occur through the transcription factor PacC (Tilburn et al., 1995), which has been shown to regulate several secondary metabolites including DON in *F. graminearum* (Merhej et al., 2011) and bikaverin in *F. fujikuroi* (Wiemann et al., 2009).

3.5. Production of fusarielins in wheat

To examine whether fusarielins are produced during infection, we inoculated seven wheat spikes with spores of *F. graminearum* (NRRL 31084, Fig. 5). Fusarielin H could be detected in all samples ranging from 392 to 1865 ng/g (mean: 989 ng/g), whereas fusarielins F and G were not produced in detectable amounts. The results show that fusarielins can be produced in infected cereals. In addition ten Danish cereal (primarily wheat) samples with high DON levels were also examined, but none of the fusarielins could be detected in these samples. These observations correlated with expression data from *F. graminearum* wheat infection assays, which show that the fusarielin gene cluster is inactive during infection (Supplementary Fig. 2). Our results from the experiment with the different carbon sources showed furthermore that starch did not induce fusarielin production, which may explain why fusarielins were not produced in infected wheat kernels. Additionally we observed that nitrate induced fusarielin production on more carbon sources than arginine, which is the opposite of DON, which is induced by arginine (Gardiner et al., 2009) and is involved in infection (Ilgen et al., 2009; Boenisch and Schafer, 2011). Thus it is unlikely that the fusarielins are required for pathogenicity and their biological role in the *Fusarium* life cycle remains unclear.

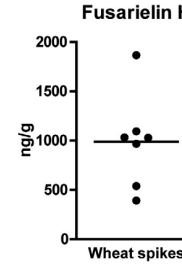

Fig. 5. Production of fusarielin H in seven wheat spikes inoculated with *F. graminearum* (NRRL 31084). Each dot represents one sample and the mean is shown as a horizontal line.

4. Conclusions

The ability to produce fusarielins was restricted to *F. graminearum* and *F. tricinctum* out of the 15 *Fusarium* species tested in the present study. The production of fusarielins was influenced by ambient conditions including nitrogen and carbon sources, time, temperature and pH. Fusarielin H, the predominant fusarielin in *F. graminearum*, was also found in artificially inoculated wheat spikes. However the low concentration does not suggest a role in plant pathogenicity.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ijfoodmicro.2012.10.016.

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2009, 28.–29.10.	Biodiversity of grasslang-based farming systems in Europe. EU FP.7. projekt "FLAVOURE" kursus, Saku, Eesti.
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2010, 29.11.–17.12.	Täiendõpe Aarhusi Ülikoolis, Põllumajandusteaduste teaduskond, Loomade tervise ja Bioteaduste osakond, Taani. EU FP.7. projekt "FLAVOURE".
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2005–2008	EV Põllumajandusministeeriumi projekt Baas 1: „Põllu-ja rohumaakultuuride saagi kvantiteedi ja kvaliteedi optimeerimine agrotehniliste võtete ja erinevate tehnoloogiate integreerimisel“, põhitäitja.
2005–2008	Projekt nr. ETF6154 : „Eesti teravilja nakatatus hallituseentest ning selle sõltuvus agroökoloogilistest teguritest “, põhitäitja.
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2006–2010	EV Põllumajandusministeeriumi projekt nr. P-1.2: „Tera- ja kaunviljade ning õlikultuuride toidu- ja söödakvaliteedi parandamise võimaluste selgitamine, rakendades majanduslikult efektiivseid ning keskkonna-säästlikke agrotehnoloogilisi meetmeid“, põhitäitja.
2008–2011	EV Põllumajandusministeeriumi projekt nr. 1.6: „Teaduselt tootmisele“, põhitäitja.
2008–2012	EV Põllumajandusministeeriumi projekt nr. P0112: „Katseasjanduse nõukogu töö korraldamine“, põhitäitja.

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2012–2014	EV Põllumajandusministeeriumi projekt nr. 50401018: „Erinevate viljelusmeetodite (sh otsekülv) rakendusteaduslik kompleksuuring“, põhitäitja.
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LIST OF PUBLICATIONS

1.1. Publications indexed in the ISI Web of Science database:

Karron, E., Runno-Paurson, E., Lõiveke, H., Islamov, B., Edesi, L., Kütt, M.-L., Talve, T., Lauringson, E., Hõrak, H., Niinemets, Ü. 2020. Application of widely used fungicides does not necessarily affect grain yield, and incidence of *Fusarium* spp. and mycotoxins DON, HT-2 and T-2 in spring barley in northern climates. *Kvasny Prumysl*, 66 (1), 215−223. doi: 10.188832/kp2020.66.215.

Edesi, L., Talve, T., **Akk, E.,** Võsa, T., Saue, T., Loide, V., Vettik, R., Plakk, T., Tamm, K. 2020. Effects of acidified pig slurry application in winter wheat (*Triticum aestivum* L.) field trial on soil chemical and microbiological properties. *Soil & Tillage*, 202, 104650. doi: 104650.10.1016/j.still.2020.104650.

Edesi, L., Kangor, T., Loide, V., Vettik, R., Tamm, I., Kennedy, H.J., Haljak, M., Tamm, Ü., Võsa, T., Tamm, K., Talve, T., **Karron, E.** 2020. Effects of lake sediment on soil chemical composition, dehydrogenase activity and grain yield and quality in organic oats and spring barley succession. *Agronomy Research*, 18 (3), 2022−2032. doi: 10.15159/AR.20.197.

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Edesi, L., Järvan, M., Noormets, M., Lauringson, E., Adamson, A., **Akk, E**. 2012. The importance of solid cattle manure application on soil microorganisms in organic and conventional cultivation. *Acta Agriculturæ Scandinavica*, Section B - Soil & Plant Science, 583–594. doi: 10.1080/09064710.2012.678380.

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Edesi, L., Järvan, M., Lauringson, E., **Akk, E.,** Tamm, K. 2013. The effect of solid cattle manure on soil microbial activity and on plate count microorganisms in organic and conventional farming systems. *International Journal of Current Microbiology and Applied Sciences*, 12, 476–488.

Akk, E., Lõiveke, H., Edesi, L., Kütt, M.-L., Lauringson, E., Kastanje, V. 2013. Formation of the abundance of microfungi on the barley grain grown as pure and mixed crops in Central and North Estonia. *Estonian Journal of Ecology*, 62, 265–275.

Ilumäe, E., **Akk, E.,** Hansson, A., Kastanje, V. 2009. Changes the content of organic matter in soil during the whole cycle of crop rotation. *Agronomy Research*, 263–268.

3.2. Articles/chapters in books published by the publishers not listed in Annex:

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3.5. Articles/presentations published in local conference proceedings:

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Lõiveke, H., Lättemäe, P., **Akk, E**. 2013. Söödad vajavad kontrollimist. *Maamajandus,* nr 5, 11.

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6.6. Articles in other journals and newspapers:

Lõiveke, H., **Akk, E.,** Puusepp, P. 2014. Teraviljaseeme külviks valmis. *Maaleht*, 18.04.

Lõiveke, H., **Akk, E**. 2014. Kahjureid ja lesti tuleb korralikult tõrjuda. *Maaleht* 12.09.

Lõiveke, H., **Akk, E**. 2011. Ohutu teravilja nimel. *Maaleht. Messileht*, 8–9.

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